

**Diagnostics and Therapeutics for Diseases Associated with Peroxisome Proliferative Activated Receptor alpha (PPARA)**

**Technical field of the invention**

The present invention is in the field of molecular biology, more particularly, the present invention  
5 relates to nucleic acid sequences and amino acid sequences of a human PPAR $\alpha$  and its regulation for the treatment of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in mammals.

**Background of the invention**

10 **Nuclear Receptors**

PPAR $\alpha$  belongs to the superfamily of nuclear receptors (NR) [[Robinson-Rechavi et al., 2003], [Sher et al. (1993)], [Kersten et al. (2000)], US 6544743, WO0120037, US 6689574] which forms a related but diverse array of ligand-modulated transcription factors that control diverse aspects of growth, development and homeostasis [Olefsky & Jerrold, 2001]. The family comprises the  
15 nuclear hormone receptors for which hormonal ligands have been identified, and orphan receptors with still unknown ligands.

The ligands of NRs are generally lipophilic because they traverse the plasma membrane to bind to their cognate receptors that are located in the cytoplasm or in the nucleus.

Six structural domains A-F (from N- to C-terminus) of NR are known [Bourguet et al. 2000]. All  
20 of the NRs have common structural features which include a central DNA binding domain (DBD, domain C) responsible for targeting the receptor to a specific DNA sequence, a ligand binding domain (LBD, domain E), that binds to specific ligands and activates transcription and a hinge region (domain D) connecting DBD and LBD. The N-terminus is formed by the A/B domain comprising a ligand-independent activation domain. The C-terminal F domain, the function of  
25 which is poorly understood, is not present in all receptors.

NRs activate transcription as homo- or heterodimers by binding to specific response elements (REs). The REs exist of two half-sites with different spacings, forming inverted or direct repeats. Based on their mode of action four different classes of NRs have been defined: class 1 is formed by the known steroid hormone receptors, that act as homodimers and bind to inverted repeats; class  
30 2 includes receptors forming heterodimers with RXR; class 3 contains homodimeric NRs binding

to direct repeats and class 4 is formed by NRs that bind as monomers to single half-sites. Classes 3 and 4 contain only orphan receptors.

NRs act as transcription activators in complex with co-activators. Ligand binding to NRs induces a conformational change which allows recruitment of specific co-activators. In the absence of ligand 5 some receptors also act as transcriptional repressors in complex with co-repressors.

The fact that steroid hormone receptors belong to the family of nuclear receptors indicates that these receptors have an established, proven history as therapeutic targets. Examples for marketed pharmaceuticals based on steroid receptor ligands comprise preparations containing oestrogens, coticosteroids or mineralocorticoids. Clearly, there is a need for identification and charac-

10 terization of further receptors which can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, infections such as bacterial, fungal, protozoan, and viral infections, particularly those caused by HIV viruses, cancers, allergies including asthma, cardiovascular diseases including acute heart failure, hypotension, hypertension, angina pectoris, myocardial infarction, hematological diseases, genito-urinary diseases including 15 urinary incontinence and benign prostate hyperplasia, osteoporosis, peripheral and central nervous system disorders including pain, Alzheimer's disease and Parkinson's disease, respiratory diseases, metabolic diseases, inflammatory diseases, gastro-enterological diseases, diseases of the endocrine system, dermatological diseases, diseases of muscles or the skeleton, immunological diseases, developmental diseases or diseases of the reproductive system.

## 20 **TaqMan-Technology / expression profiling**

TaqMan is a recently developed technique, in which the release of a fluorescent reporter dye from a hybridisation probe in real-time during a polymerase chain reaction (PCR) is proportional to the accumulation of the PCR product. Quantification is based on the early, linear part of the reaction, and by determining the threshold cycle (CT), at which fluorescence above background is first 25 detected.

Gene expression technologies may be useful in several areas of drug discovery and development, such as target identification, lead optimization, and identification of mechanisms of action. The TaqMan technology can be used to compare differences between expression profiles of normal tissue and diseased tissue. Expression profiling has been used in identifying genes, which are up- 30 or downregulated in a variety of diseases. An interesting application of expression profiling is temporal monitoring of changes in gene expression during disease progression and drug treatment or in patients versus healthy individuals. The premise in this approach is that changes in pattern of gene expression in response to physiological or environmental stimuli (e.g., drugs) may serve as

indirect clues about disease-causing genes or drug targets. Moreover, the effects of drugs with established efficacy on global gene expression patterns may provide a guidepost, or a genetic signature, against which a new drug candidate can be compared.

### PPARA

5 The nucleotide sequence of PPARA is accessible in the databases by the accession number NM\_005036 and is given in SEQ ID NO: 1. The amino acid sequence of PPARA depicted in SEQ ID NO: 2.

Nuclear hormone receptors form one evolutionary related super-family of proteins, which mediate the interaction between hormones (or other ligands) and gene expression in animals. Early 10 phylogenetic analyses showed two main periods of gene duplication which gave rise to present-day diversity in most animals: one at the origin of the family, and another specifically in vertebrates. Moreover this second period is composed itself by, probably, two rounds of duplication at the origin of vertebrates. There are indeed often two, three or four vertebrate orthologs of each 15 invertebrate nuclear receptor, in accordance with this theory. The complete genome of *Drosophila melanogaster* contains 21 nuclear receptors, compared to 49 in the human genome [Robinson-Rechavi et al., 2003].

Peroxisome proliferators are a diverse group of chemicals which includes hypolipidemic drugs, herbicides, leukotriene antagonists, and plasticizers. Two major categories of peroxisome proliferator chemicals play a significant role in current society: the fibrate class of hypolipidemic 20 drugs, which are used to reduce triglycerides and cholesterol in patients with hyperlipidemia; and phthalate ester plasticizers, which are used in the production of highly versatile, flexible vinyl plastics. Peroxisome proliferators induce hepatomegaly as a result of liver hyperplasia and an increase in the size and number of peroxisomes. Sher et al. [Sher et al. (1993)] cloned a cDNA for 25 human peroxisome proliferator-activated receptor from a human liver cDNA library. The PPAR cDNA exhibited 85% and 91% DNA and deduced amino acid sequence identity, respectively, with mouse PPAR.

Kersten et al. [Kersten et al. (2000)] reviewed the roles of PPARs in health and disease.

PPARA is published in patents US 6544743, WO0120037 and US 6689574.

### Summary of the invention

30 The invention relates to novel disease associations of PPARA polypeptides and polynucleotides. The invention also relates to novel methods of screening for therapeutic agents for the treatment of

cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal. The invention also relates to pharmaceutical compositions for the treatment of cardiovascular diseases, 5 gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a PPARA polypeptide, a PPARA polynucleotide, or regulators of PPARA or modulators of PPARA activity. The invention further comprises methods of diagnosing cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal.

10 **Brief Description of the Drawings**

Fig. 1 shows the nucleotide sequence of a PPARA receptor polynucleotide (SEQ ID NO: 1).

Fig. 2 shows the amino acid sequence of a PPARA receptor polypeptide (SEQ ID NO: 2).

Fig. 3 shows the nucleotide sequence of a primer useful for the invention (SEQ ID NO: 3).

Fig. 4 shows the nucleotide sequence of a primer useful for the invention (SEQ ID NO: 4).

15 Fig. 5 shows a nucleotide sequence useful as a probe to detect proteins of the invention (SEQ ID NO: 5).

**Detailed description of the invention**

**Definition of terms**

An “oligonucleotide” is a stretch of nucleotide residues which has a sufficient number of bases to 20 be used as an oligomer, amplimer or probe in a polymerase chain reaction (PCR). Oligonucleotides are prepared from genomic or cDNA sequence and are used to amplify, reveal, or confirm the presence of a similar DNA or RNA in a particular cell or tissue. Oligonucleotides or oligomers comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 35 nucleotides, preferably about 25 nucleotides.

25 “Probes” may be derived from naturally occurring or recombinant single- or double-stranded nucleic acids or may be chemically synthesized. They are useful in detecting the presence of identical or similar sequences. Such probes may be labeled with reporter molecules using nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Nucleic acid probes may be used in southern, northern or in situ hybridizations to determine whether DNA or 30 RNA encoding a certain protein is present in a cell type, tissue, or organ.

A "fragment of a polynucleotide" is a nucleic acid that comprises all or any part of a given nucleotide molecule, the fragment having fewer nucleotides than about 6 kb, preferably fewer than about 1 kb.

"Reporter molecules" are radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents which associate with a particular nucleotide or amino acid sequence, thereby establishing the presence of a certain sequence, or allowing for the quantification of a certain sequence.

"Chimeric" molecules may be constructed by introducing all or part of the nucleotide sequence of this invention into a vector containing additional nucleic acid sequence which might be expected to change any one or several of the following PPARA characteristics: cellular location, distribution, 10 ligand-binding affinities, interchain affinities, degradation/turnover rate, signaling, etc.

"Active", with respect to a PPARA polypeptide, refers to those forms, fragments, or domains of a PPARA polypeptide which retain the biological and/or antigenic activity of a PPARA polypeptide.

"Naturally occurring PPARA polypeptide" refers to a polypeptide produced by cells which have not been genetically engineered and specifically contemplates various polypeptides arising from 15 post-translational modifications of the polypeptide including but not limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

"Derivative" refers to polypeptides which have been chemically modified by techniques such as ubiquitination, labeling (see above), pegylation (derivatization with polyethylene glycol), and chemical insertion or substitution of amino acids such as ornithine which do not normally occur in 20 human proteins.

"Conservative amino acid substitutions" result from replacing one amino acid with another having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation 25 allowed may be experimentally determined by producing the peptide synthetically while systematically making insertions, deletions, or substitutions of nucleotides in the sequence using recombinant DNA techniques.

A "signal sequence" or "leader sequence" can be used, when desired, to direct the polypeptide through a membrane of a cell. Such a sequence may be naturally present on the polypeptides of 30 the present invention or provided from heterologous sources by recombinant DNA techniques.

An "oligopeptide" is a short stretch of amino acid residues and may be expressed from an oligonucleotide. Oligopeptides comprise a stretch of amino acid residues of at least 3, 5, 10 amino acids and at most 10, 15, 25 amino acids, typically of at least 9 to 13 amino acids, and of sufficient length to display biological and/or antigenic activity.

5 "Inhibitor" is any substance which retards or prevents a chemical or physiological reaction or response. Common inhibitors include but are not limited to antisense molecules, antibodies, and antagonists.

10 "Standard expression" is a quantitative or qualitative measurement for comparison. It is based on a statistically appropriate number of normal samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles.

"Animal" as used herein may be defined to include human, domestic (e.g., cats, dogs, etc.), agricultural (e.g., cows, horses, sheep, etc.) or test species (e.g., mouse, rat, rabbit, etc.).

A "PPARA polynucleotide", within the meaning of the invention, shall be understood as being a nucleic acid molecule selected from a group consisting of

15 (i) nucleic acid molecules encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 2,

(ii) nucleic acid molecules comprising the sequence of SEQ ID NO: 1,

(iii) nucleic acid molecules having the sequence of SEQ ID NO: 1,

(iv) nucleic acid molecules the complementary strand of which hybridizes under stringent 20 conditions to a nucleic acid molecule of (i), (ii), or (iii); and

(v) nucleic acid molecules the sequence of which differs from the sequence of a nucleic acid molecule of (iii) due to the degeneracy of the genetic code;

wherein the polypeptide encoded by said nucleic acid molecule has PPARA activity.

25 A "PPARA polypeptide", within the meaning of the invention, shall be understood as being a polypeptide selected from a group consisting of

(i) polypeptides having the sequence of SEQ ID NO: 2,

(ii) polypeptides comprising the sequence of SEQ ID NO: 2,

(iii) polypeptides encoded by PPARA polynucleotides; and

(iv) polypeptides which show at least 99%, 98%, 95%, 90%, or 80% homology with a polypeptide of (i), (ii), or (iii);

wherein said polypeptide has PPARA activity.

The nucleotide sequences encoding a PPARA (or their complement) have numerous applications

5 in techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use in the construction of oligomers for PCR, use for chromosome and gene mapping, use in the recombinant production of PPARA, and use in generation of antisense DNA or RNA, their chemical analogs and the like. Uses of nucleotides encoding a PPARA disclosed herein are exemplary of known techniques and are not intended to limit their use in any  
10 technique known to a person of ordinary skill in the art. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, e.g., the triplet genetic code, specific base pair interactions, etc.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic

15 code, a multitude of PPARA - encoding nucleotide sequences may be produced. Some of these will only bear minimal homology to the nucleotide sequence of the known and naturally occurring PPARA. The invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as  
20 applied to the nucleotide sequence of naturally occurring PPARA, and all such variations are to be considered as being specifically disclosed.

Although the nucleotide sequences which encode a PPARA, its derivatives or its variants are

preferably capable of hybridizing to the nucleotide sequence of the naturally occurring PPARA

polynucleotide under stringent conditions, it may be advantageous to produce nucleotide

25 sequences encoding PPARA polypeptides or its derivatives possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding a PPARA polypeptide and/or its derivatives without altering the  
30 encoded amino acid sequence include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

Nucleotide sequences encoding a PPARA polypeptide may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques. Useful nucleotide sequences for joining to PPARA polynucleotides include an assortment of cloning vectors such as plasmids, cosmids, lambda phage derivatives, phagemids, and the like. Vectors of 5 interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, etc. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for one or more host cell systems.

Another aspect of the subject invention is to provide for PPARA-specific hybridization probes 10 capable of hybridizing with naturally occurring nucleotide sequences encoding PPARA. Such probes may also be used for the detection of similar Nuclear Receptors encoding sequences and should preferably show at least 40% nucleotide identity to PPARA polynucleotides. The hybridization probes of the subject invention may be derived from the nucleotide sequence presented as SEQ ID NO: 1 or from genomic sequences including promoter, enhancers or introns 15 of the native gene. Hybridization probes may be labelled by a variety of reporter molecules using techniques well known in the art.

It will be recognized that many deletional or mutational analogs of PPARA polynucleotides will be 20 effective hybridization probes for PPARA polynucleotides. Accordingly, the invention relates to nucleic acid sequences that hybridize with such PPARA encoding nucleic acid sequences under stringent conditions.

“Stringent conditions“ refers to conditions that allow for the hybridization of substantially related nucleic acid sequences. For instance, such conditions will generally allow hybridization of sequence with at least about 85% sequence identity, preferably with at least about 90% sequence 25 identity, more preferably with at least about 95% sequence identity. Hybridization conditions and probes can be adjusted in well-characterized ways to achieve selective hybridization of human-derived probes. Stringent conditions, within the meaning of the invention are 65°C in a buffer containing 1 mM EDTA, 0.5 M NaHPO<sub>4</sub> (pH 7.2), 7 % (w/v) SDS.

Nucleic acid molecules that will hybridize to PPARA polynucleotides under stringent conditions can be identified functionally. Without limitation, examples of the uses for hybridization probes 30 include: histochemical uses such as identifying tissues that express PPARA; measuring mRNA levels, for instance to identify a sample's tissue type or to identify cells that express abnormal levels of PPARA; and detecting polymorphisms of PPARA.

PCR provides additional uses for oligonucleotides based upon the nucleotide sequence which encodes PPARA. Such probes used in PCR may be of recombinant origin, chemically synthesized, or a mixture of both. Oligomers may comprise discrete nucleotide sequences employed under optimized conditions for identification of PPARA in specific tissues or diagnostic use. The same 5 two oligomers, a nested set of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for identification of closely related DNAs or RNAs.

Rules for designing polymerase chain reaction (PCR) primers are now established, as reviewed by PCR Protocols. Degenerate primers, i.e., preparations of primers that are heterogeneous at given sequence locations, can be designed to amplify nucleic acid sequences that are highly homologous 10 to, but not identical with PPARA. Strategies are now available that allow for only one of the primers to be required to specifically hybridize with a known sequence. For example, appropriate nucleic acid primers can be ligated to the nucleic acid sought to be amplified to provide the hybridization partner for one of the primers. In this way, only one of the primers need be based on the sequence of the nucleic acid sought to be amplified.

15 PCR methods for amplifying nucleic acid will utilize at least two primers. One of these primers will be capable of hybridizing to a first strand of the nucleic acid to be amplified and of priming enzyme-driven nucleic acid synthesis in a first direction. The other will be capable of hybridizing the reciprocal sequence of the first strand (if the sequence to be amplified is single stranded, this sequence will initially be hypothetical, but will be synthesized in the first amplification cycle) and 20 of priming nucleic acid synthesis from that strand in the direction opposite the first direction and towards the site of hybridization for the first primer. Conditions for conducting such amplifications, particularly under preferred stringent hybridization conditions, are well known.

Other means of producing specific hybridization probes for PPARA include the cloning of nucleic 25 acid sequences encoding PPARA or PPARA derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate reporter molecules.

It is possible to produce a DNA sequence, or portions thereof, entirely by synthetic chemistry. After synthesis, the nucleic acid sequence can be inserted into any of the many available DNA 30 vectors and their respective host cells using techniques which are well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into the nucleotide sequence. Alternately, a portion of sequence in which a mutation is desired can be synthesized and recombined with longer portion of an existing genomic or recombinant sequence.

PPARA polynucleotides may be used to produce a purified oligo- or polypeptide using well known methods of recombinant DNA technology. The oligopeptide may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which the nucleotide sequence was derived or from a different species. Advantages of producing an 5 oligonucleotide by recombinant DNA technology include obtaining adequate amounts of the protein for purification and the availability of simplified purification procedures.

#### Quantitative determinations of nucleic acids

An important step in the molecular genetic analysis of human disease is often the enumeration of the copy number of a nucleic acid or the relative expression of a gene in particular tissues.

10 Several different approaches are currently available to make quantitative determinations of nucleic acids. Chromosome-based techniques, such as comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH) facilitate efforts to cytogenetically localize genomic regions that are altered in tumor cells. Regions of genomic alteration can be narrowed further using loss of heterozygosity analysis (LOH), in which disease DNA is analyzed and compared with 15 normal DNA for the loss of a heterozygous polymorphic marker. The first experiments used restriction fragment length polymorphisms (RFLPs) [Johnson, (1989)], or hypervariable minisatellite DNA [Barnes, 2000]. In recent years LOH has been performed primarily using PCR amplification of microsatellite markers and electrophoresis of the radio labelled [Jeffreys, (1985)] or fluorescently labelled PCR products [Weber, (1990)] and compared between paired normal and 20 disease DNAs.

A number of other methods have also been developed to quantify nucleic acids [Gergen, (1992)]. More recently, PCR and RT-PCR methods have been developed which are capable of measuring the amount of a nucleic acid in a sample. One approach, for example, measures PCR product quantity in the log phase of the reaction before the formation of reaction products plateaus 25 [Thomas, (1980)].

A gene sequence contained in all samples at relatively constant quantity is typically utilized for sample amplification efficiency normalization. This approach, however, suffers from several drawbacks. The method requires that each sample has equal input amounts of the nucleic acid and that the amplification efficiency between samples is identical until the time of analysis. 30 Furthermore, it is difficult using the conventional methods of PCR quantitation such as gel electrophoresis or plate capture hybridization to determine that all samples are in fact analyzed during the log phase of the reaction as required by the method.

Another method called quantitative competitive (QC)-PCR, as the name implies, relies on the inclusion of an internal control competitor in each reaction [Piatak, (1993), BioTechniques]. The efficiency of each reaction is normalized to the internal competitor. A known amount of internal competitor is typically added to each sample. The unknown target PCR product is compared with 5 the known competitor PCR product to obtain relative quantitation. A difficulty with this general approach lies in developing an internal control that amplifies with the same efficiency than the target molecule.

#### *5' Fluorogenic Nuclease Assays*

Fluorogenic nuclease assays are a real time quantitation method that uses a probe to monitor 10 formation of amplification product. The basis for this method of monitoring the formation of amplification product is to measure continuously PCR product accumulation using a dual-labelled fluorogenic oligonucleotide probe, an approach frequently referred to in the literature simply as the "TaqMan method" [Piatak, (1993), Science; Heid, (1996); Gibson, (1996); Holland. (1991)].

The probe used in such assays is typically a short (about 20-25 bases) oligonucleotide that is 15 labeled with two different fluorescent dyes. The 5' terminus of the probe is attached to a reporter dye and the 3' terminus is attached to a quenching dye, although the dyes could be attached at other locations on the probe as well. The probe is designed to have at least substantial sequence complementarity with the probe binding site. Upstream and downstream PCR primers which bind to flanking regions of the locus are added to the reaction mixture. When the probe is intact, energy 20 transfer between the two fluorophors occurs and the quencher quenches emission from the reporter. During the extension phase of PCR, the probe is cleaved by the 5' nuclease activity of a nucleic acid polymerase such as Taq polymerase, thereby releasing the reporter from the oligonucleotide-quencher and resulting in an increase of reporter emission intensity which can be measured by an appropriate detector.

25 One detector which is specifically adapted for measuring fluorescence emissions such as those created during a fluorogenic assay is the ABI 7700 or 4700 HT manufactured by Applied Biosystems, Inc. in Foster City, Calif. The ABI 7700 uses fiber optics connected with each well in a 96-or 384 well PCR tube arrangement. The instrument includes a laser for exciting the labels and is capable of measuring the fluorescence spectra intensity from each tube with continuous 30 monitoring during PCR amplification. Each tube is re-examined every 8.5 seconds.

Computer software provided with the instrument is capable of recording the fluorescence intensity of reporter and quencher over the course of the amplification. The recorded values will then be used to calculate the increase in normalized reporter emission intensity on a continuous basis. The

increase in emission intensity is plotted versus time, i.e., the number of amplification cycles, to produce a continuous measure of amplification. To quantify the locus in each amplification reaction, the amplification plot is examined at a point during the log phase of product accumulation. This is accomplished by assigning a fluorescence threshold intensity above background  
5 and determining the point at which each amplification plot crosses the threshold (defined as the threshold cycle number or Ct). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube. Assuming that each reaction functions at 100% PCR efficiency, a difference of one Ct represents a two-fold difference in the amount of starting template. The fluorescence value can be used in conjunction with a standard curve to  
10 determine the amount of amplification product present.

#### *Non-Probe-Based Detection Methods*

A variety of options are available for measuring the amplification products as they are formed. One method utilizes labels, such as dyes, which only bind to double stranded DNA. In this type of approach, amplification product (which is double stranded) binds dye molecules in solution to  
15 form a complex. With the appropriate dyes, it is possible to distinguish between dye molecules free in solution and dye molecules bound to amplification product. For example, certain dyes fluoresce only when bound to amplification product. Examples of dyes which can be used in methods of this general type include, but are not limited to, Syber Green.TM. and Pico Green from Molecular Probes, Inc. of Eugene, Oreg., ethidium bromide, propidium iodide, chromomycin,  
20 acridine orange, Hoechst 33258, Toto-1, Yoyo-1, DAPI (4',6-diamidino-2-phenylindole hydrochloride).

Another real time detection technique measures alteration in energy fluorescence energy transfer between fluorophors conjugated with PCR primers [Livak, (1995)].

#### *Probe-Based Detection Methods*

25 These detection methods involve some alteration to the structure or conformation of a probe hybridized to the locus between the amplification primer pair. In some instances, the alteration is caused by the template-dependent extension catalyzed by a nucleic acid polymerase during the amplification process. The alteration generates a detectable signal which is an indirect measure of the amount of amplification product formed.  
30 For example, some methods involve the degradation or digestion of the probe during the extension reaction. These methods are a consequence of the 5'-3' nuclease activity associated with some nucleic acid polymerases. Polymerases having this activity cleave mononucleotides or small

oligonucleotides from an oligonucleotide probe annealed to its complementary sequence located within the locus.

The 3' end of the upstream primer provides the initial binding site for the nucleic acid polymerase. As the polymerase catalyzes extension of the upstream primer and encounters the bound probe, the 5 nucleic acid polymerase displaces a portion of the 5' end of the probe and through its nuclease activity cleaves mononucleotides or oligonucleotides from the probe.

The upstream primer and the probe can be designed such that they anneal to the complementary strand in close proximity to one another. In fact, the 3' end of the upstream primer and the 5' end of the probe may abut one another. In this situation, extension of the upstream primer is not 10 necessary in order for the nucleic acid polymerase to begin cleaving the probe. In the case in which intervening nucleotides separate the upstream primer and the probe, extension of the primer is necessary before the nucleic acid polymerase encounters the 5' end of the probe. Once contact occurs and polymerization continues, the 5'-3' exonuclease activity of the nucleic acid polymerase begins cleaving mononucleotides or oligonucleotides from the 5' end of the probe. Digestion of 15 the probe continues until the remaining portion of the probe dissociates from the complementary strand.

In solution, the two end sections can hybridize with each other to form a hairpin loop. In this conformation, the reporter and quencher dye are in sufficiently close proximity that fluorescence from the reporter dye is effectively quenched by the quencher dye. Hybridized probe, in contrast, 20 results in a linearized conformation in which the extent of quenching is decreased. Thus, by monitoring emission changes for the two dyes, it is possible to indirectly monitor the formation of amplification product.

### *Probes*

The labeled probe is selected so that its sequence is substantially complementary to a segment of 25 the test locus or a reference locus. As indicated above, the nucleic acid site to which the probe binds should be located between the primer binding sites for the upstream and downstream amplification primers.

### *Primers*

The primers used in the amplification are selected so as to be capable of hybridizing to sequences 30 at flanking regions of the locus being amplified. The primers are chosen to have at least substantial complementarity with the different strands of the nucleic acid being amplified. When a

probe is utilized to detect the formation of amplification products, the primers are selected in such that they flank the probe, i.e. are located upstream and downstream of the probe.

The primer must have sufficient length so that it is capable of priming the synthesis of extension products in the presence of an agent for polymerization. The length and composition of the primer depends on many parameters, including, for example, the temperature at which the annealing reaction is conducted, proximity of the probe binding site to that of the primer, relative concentrations of the primer and probe and the particular nucleic acid composition of the probe. Typically the primer includes 15-30 nucleotides. However, the length of the primer may be more or less depending on the complexity of the primer binding site and the factors listed above.

10 *Labels for Probes and Primers*

The labels used for labeling the probes or primers of the current invention and which can provide the signal corresponding to the quantity of amplification product can take a variety of forms. As indicated above with regard to the 5' fluorogenic nuclease method, a fluorescent signal is one signal which can be measured. However, measurements may also be made, for example, by monitoring radioactivity, colorimetry, absorption, magnetic parameters, or enzymatic activity. Thus, labels which can be employed include, but are not limited to, fluorophors, chromophores, radioactive isotopes, electron dense reagents, enzymes, and ligands having specific binding partners (e.g., biotin-avidin).

Monitoring changes in fluorescence is a particularly useful way to monitor the accumulation of amplification products. A number of labels useful for attachment to probes or primers are commercially available including fluorescein and various fluorescein derivatives such as FAM, HEX, TET and JOE (all which are available from Applied Biosystems, Foster City, Calif.); lucifer yellow, and coumarin derivatives.

Labels may be attached to the probe or primer using a variety of techniques and can be attached at the 5' end, and/or the 3' end and/or at an internal nucleotide. The label can also be attached to spacer arms of various sizes which are attached to the probe or primer. These spacer arms are useful for obtaining a desired distance between multiple labels attached to the probe or primer.

In some instances, a single label may be utilized; whereas, in other instances, such as with the 5' fluorogenic nuclease assays for example, two or more labels are attached to the probe. In cases wherein the probe includes multiple labels, it is generally advisable to maintain spacing between the labels which is sufficient to permit separation of the labels during digestion of the probe through the 5'-3' nuclease activity of the nucleic acid polymerase.

### **Patients Exhibiting Symptoms of Disease**

A number of diseases are associated with changes in the copy number of a certain gene. For patients having symptoms of a disease, the real-time PCR method can be used to determine if the patient has copy number alterations which are known to be linked with diseases that are associated with the symptoms the patient has.

### **PPARA expression**

#### *PPARA fusion proteins*

Fusion proteins are useful for generating antibodies against PPARA polypeptides and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions of PPARA polypeptides. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A PPARA fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment can comprise at least 54, 75, 100, 125, 139, 150, 175, 200, 225, 250, or 275 contiguous amino acids of SEQ ID NO: 2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length PPARA.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include, but are not limited to  $\beta$  galactosidase,  $\beta$ -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, herpes simplex virus (HSV) BP16 protein fusions and G-protein fusions (for example G(alpha)16, Gs, Gi). A fusion protein also can be engineered to contain a cleavage site located adjacent to the PPARA.

*Preparation of Polynucleotides*

A naturally occurring PPARA polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated PPARA polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments which comprise PPARA nucleotide sequences. Isolated polynucleotides are in preparations which are free or at least 70, 80, or 90% free of other molecules.

PPARA cDNA molecules can be made with standard molecular biology techniques, using PPARA mRNA as a template. PPARA cDNA molecules can thereafter be replicated using molecular biology techniques known in the art. An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize PPARA polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode PPARA having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

20 *Extending Polynucleotides*

Various PCR-based methods can be used to extend nucleic acid sequences encoding human PPARA, for example to detect upstream sequences of PPARA gene such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus. Genomic DNA is first amplified in the presence of a primer 25 to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR also can be used to amplify or extend sequences using divergent primers based on a 30 known region. Primers can be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at

temperatures about 68-72°C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which can be used is capture PCR, which involves PCR amplification of DNA 5 fragments adjacent to a known sequence in human and yeast artificial chromosome DNA. In this method, multiple restriction enzyme digestions and ligations also can be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected 10 to include larger cDNAs. Randomly-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries can be useful for extension of sequence into 5' non-transcribed regulatory regions.

15 Commercially available capillary electrophoresis systems can be used to analyze the size or confirm the nucleotide sequence of PCR or sequencing products. For example, capillary sequencing can employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity can be converted to electrical signal 20 using appropriate equipment and software (e.g., GENOTYPER and Sequence NAVIGATOR, Perkin Elmer), and the entire process from loading of samples to computer analysis and electronic data display can be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

25 *Obtaining Polypeptides*

PPARA can be obtained, for example, by purification from human cells, by expression of PPARA polynucleotides, or by direct chemical synthesis.

*Protein Purification*

PPARA can be purified from any human cell which expresses the receptor, including those which 30 have been transfected with expression constructs which express PPARA. A purified PPARA is separated from other compounds which normally associate with PPARA in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but

are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

*Expression of PPARA Polynucleotides*

To express PPARA, PPARA polynucleotides can be inserted into an expression vector which

5 contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding PPARA and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination.

10 A variety of expression vector/host systems can be utilized to contain and express sequences encoding PPARA. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, 15 cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of the vector - enhancers, promoters, 5' and 3' untranslated regions -- which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. 20 Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. 25 Promoters or enhancers derived from the genomes of plant cells (*e.g.*, heat shock, RUBISCO, and storage protein genes) or from plant viruses (*e.g.*, viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple 30 copies of a nucleotide sequence encoding PPARA, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

*Bacterial and Yeast Expression Systems*

In bacterial systems, a number of expression vectors can be selected. For example, when a large quantity of PPARA is needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified can be used. Such vectors include, but are

5 not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). In a BLUESCRIPT vector, a sequence encoding PPARA can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase so that a hybrid protein is produced. pIN vectors or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptides as fusion proteins with 10 glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

15 *Plant and Insect Expression Systems*

If plant expression vectors are used, the expression of sequences encoding PPARA can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV. Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock 20 promoters can be used. These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection.

An insect system also can be used to express PPARA. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding PPARA can 25 be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of PPARA will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which PPARA can be expressed.

30 *Mammalian Expression Systems*

A number of viral-based expression systems can be used to express PPARA in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding PPARA

can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing PPARA in infected host cells [Engelhard, 1994]. If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) 5 enhancer, can be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are constructed and delivered to cells via conventional delivery methods (e.g., liposomes, polycationic amino polymers, or vesicles). Specific initiation signals also can be used to achieve more efficient translation of 10 sequences encoding PPARA. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding PPARA, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation 15 codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic.

#### *Host Cells*

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences 20 or to process the expressed PPARA in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational 25 activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high-yield production of recombinant proteins. For example, cell lines which stably express PPARA can be transformed using expression vectors 30 which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced

PPARA sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase [Logan, (1984)] and adenine phosphoribosyltransferase [Wigler, (1977)] genes which can be employed in *tk* or *aprt* cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate [Lowy, (1980)], *npt* confers resistance to the aminoglycosides, neomycin and G-418 [Wigler, (1980)], and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively [Colbere-Garapin, 1981]. Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine. Visible markers such as anthocyanins,  $\beta$ -glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount of transient or stable protein expression attributable to a specific vector system

15 *Detecting Polypeptide Expression*

Although the presence of marker gene expression suggests that a PPARA polynucleotide is also present, its presence and expression may need to be confirmed. For example, if a sequence encoding PPARA is inserted within a marker gene sequence, transformed cells containing sequences which encode PPARA can be identified by the absence of marker gene function. 20 Alternatively, a marker gene can be placed in tandem with a sequence encoding PPARA under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of PPARA polynucleotide.

Alternatively, host cells which contain a PPARA polynucleotide and which express PPARA can be identified by a variety of procedures known to those of skill in the art. These procedures include, 25 but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence of a polynucleotide sequence encoding PPARA can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding PPARA. 30 Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding PPARA to detect transformants which contain a PPARA polynucleotide.

A variety of protocols for detecting and measuring the expression of PPARA, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence

activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on PPARA can be used, or a competitive binding assay can be employed.

A wide variety of labels and conjugation techniques are known by those skilled in the art and can 5 be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding PPARA include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding PPARA can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to 10 synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include 15 radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

#### *Expression and Purification of Polypeptides*

Host cells transformed with PPARA polynucleotides can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a 20 transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing PPARA polynucleotides can be designed to contain signal sequences which direct secretion of soluble PPARA through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound PPARA.

As discussed above, other constructions can be used to join a sequence encoding PPARA to a 25 nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). 30 Inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and PPARA also can be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing PPARA and 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by IMAC (immobilized metal ion affinity

chromatography) Maddox, (1983)], while the enterokinase cleavage site provides a means for purifying PPARA from the fusion protein [Porath, (1992)].

#### *Chemical Synthesis*

Sequences encoding PPARA can be synthesized, in whole or in part, using chemical methods well known in the art. Alternatively, PPARA itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques. Protein synthesis can either be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of PPARA can be separately synthesized and combined using chemical methods to produce a full-length molecule.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography. The composition of a synthetic PPARA can be confirmed by amino acid analysis or sequencing. Additionally, any portion of the amino acid sequence of PPARA can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

#### *Production of Altered Polypeptides*

As will be understood by those of skill in the art, it may be advantageous to produce PPARA polynucleotides possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences referred to herein can be engineered using methods generally known in the art to alter PPARA polynucleotides for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

#### *Antibodies*

Any type of antibody known in the art can be generated to bind specifically to an epitope of PPARA.

“Antibody” as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv, which are capable of binding an epitope of PPARA. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 5 25, or 50 amino acid. An antibody which specifically binds to an epitope of PPARA can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are 10 well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the PPARA immunogen.

Typically, an antibody which specifically binds to PPARA provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an 15 immunochemical assay. Preferably, antibodies which specifically bind to PPARA do not detect other proteins in immunochemical assays and can immunoprecipitate PPARA from solution.

PPARA can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies. If desired, PPARA can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending 20 on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (*bacilli Calmette-Guerin*) and *Corynebacterium parvum* are especially useful.

25 Monoclonal antibodies which specifically bind to PPARA can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique [Roberge, (1995)].

In addition, techniques developed for the production of “chimeric antibodies”, the splicing of 30 mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used. Monoclonal and other antibodies also can be “humanized” to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence

differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grafting of entire complementarity determining regions. Antibodies which specifically bind to PPARA can contain antigen binding sites which are either partially or fully 5 humanized, as disclosed in U.S. 5,565,332.

Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which specifically bind to PPARA. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobulin libraries. Single-chain 10 antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template. Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught. A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant 15 DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology.

Antibodies which specifically bind to PPARA also can be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific 20 binding reagents. Other types of antibodies can be constructed and used therapeutically in methods of the invention. For example, chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, also can be prepared.

25 Antibodies according to the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which PPARA is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

#### *Antisense Oligonucleotides*

30 Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be

at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of PPARA gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamide, carboxymethyl esters, carbonates, and phosphate triesters.

Modifications of PPARA gene expression can be obtained by designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the PPARA gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature [Nicholls, (1993)]. An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a PPARA polynucleotide. Antisense oligonucleotides which comprise, for example, 2, 3, 4, or 5 or more stretches of contiguous nucleotides which are precisely complementary to a PPARA polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent PPARA nucleotides, can provide sufficient targeting specificity for PPARA mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, or 8 or more nucleotides in length. Non-complementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular PPARA polynucleotide sequence. Antisense oligonucleotides can be modified without affecting their ability to hybridize to a PPARA polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholestryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3'

hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art.

### *Ribozymes*

5     Ribozymes are RNA molecules with catalytic activity [Uhlmann, (1987)]. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endo-  
10    nucleolytic cleavage of specific nucleotide sequences. The coding sequence of a PPARA polynucleotide can be used to generate ribozymes which will specifically bind to mRNA transcribed from a PPARA polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art. For example, the cleavage activity of ribozymes can be  
15    targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target RNA.

Specific ribozyme cleavage sites within a PPARA RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, 20 and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate PPARA RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. The nucleotide sequences 25 shown in SEQ ID NO: 1 and its complement provide sources of suitable hybridization region sequences. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

30    Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease PPARA expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element

or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells (U.S. 5,641,673). Ribozymes also can be engineered to provide an additional level of 5 regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

### Screening / Screening Assays

#### *Regulators*

Regulators as used herein, refer to compounds that affect the activity of a PPARA in vivo and/or in 10 vivo. Regulators can be agonists and antagonists of a PPARA polypeptide and can be compounds that exert their effect on the PPARA activity via the expression, via post-translational modifications or by other means. Agonists of PPARA are molecules which, when bound to PPARA, increase or prolong the activity of PPARA. Agonists of PPARA include proteins, nucleic acids, carbohydrates, small molecules, or any other molecule which activate PPARA. Antagonists of 15 PPARA are molecules which, when bound to PPARA, decrease the amount or the duration of the activity of PPARA. Antagonists include proteins, nucleic acids, carbohydrates, antibodies, small molecules, or any other molecule which decrease the activity of PPARA.

The term "modulate", as it appears herein, refers to a change in the activity of PPARA polypeptide. For example, modulation may cause an increase or a decrease in protein activity, binding 20 characteristics, or any other biological, functional, or immunological properties of PPARA.

As used herein, the terms "specific binding" or "specifically binding" refer to that interaction between a protein or peptide and an agonist, an antibody, or an antagonist. The interaction is dependent upon the presence of a particular structure of the protein recognized by the binding molecule (i.e., the antigenic determinant or epitope). For example, if an antibody is specific for 25 epitope "A" the presence of a polypeptide containing the epitope A, or the presence of free unlabeled A, in a reaction containing free labeled A and the antibody will reduce the amount of labeled A that binds to the antibody.

The invention provides methods (also referred to herein as "screening assays") for identifying compounds which can be used for the treatment of cardiovascular diseases, gastroenterological 30 diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases. The methods entail the identification of candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other molecules) which bind to

PPARA and/or have a stimulatory or inhibitory effect on the biological activity of PPARA or its expression and then determining which of these compounds have an effect on symptoms or diseases regarding the cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in an *in vivo* assay.

Candidate or test compounds or agents which bind to PPARA and/or have a stimulatory or inhibitory effect on the activity or the expression of PPARA are identified either in assays that employ cells which express PPARA on the cell surface (cell-based assays) or in assays with isolated PPARA (cell-free assays). The various assays can employ a variety of variants of PPARA (e.g., full-length PPARA, a biologically active fragment of PPARA, or a fusion protein which includes all or a portion of PPARA). Moreover, PPARA can be derived from any suitable mammalian species (e.g., human PPARA, rat PPARA or murine PPARA). The assay can be a binding assay entailing direct or indirect measurement of the binding of a test compound or a known PPARA ligand to PPARA. The assay can also be an activity assay entailing direct or indirect measurement of the activity of PPARA. The assay can also be an expression assay entailing direct or indirect measurement of the expression of PPARA mRNA or PPARA protein. The various screening assays are combined with an *in vivo* assay entailing measuring the effect of the test compound on the symptoms of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

Determining the ability of the test compound to modulate the activity of PPARA can be accomplished, for example, by determining the ability of PPARA to bind to or interact with a target molecule. The target molecule can be a molecule with which PPARA binds or interacts with in nature, for example, a molecule from the extracellular milieu able to traverse the cytoplasmic membrane or a cytoplasmic molecule. The target molecule can be a component of a signal transduction pathway which facilitates transduction of an extracellular signal (e.g., a signal generated by another cell) through the cell membrane and into the cell. The target PPARA molecule can be, for example, a second intracellular protein which has catalytic activity, a protein which facilitates the association of downstream signaling molecules with PPARA or the target DNA of the nuclear receptor.

#### Nuclear receptor assays

In general two modes exist to determine ligand dependent NR activity in cell based assays. Cells that endogenously express a certain NR can be transfected with a reporter construct expressing a reporter gene (e.g. firefly luciferase) under control of a responsive promoter (Gutendorf et al.,

2001). Alternatively cells lacking an endogenous NR are co-transfected with a reporter construct and a NR expressing vector (Berger et al., 1992).

In a second approach a fusion protein of the NRs LBD and a non-mammalian DBD, that targets a corresponding promoter with a downstream reporter gene, are used. DBD from the bacterial LexA repressor or the yeast Gal4 transcription activator are described (Lee et al., 1998; Charles et al., 5 2000). The corresponding reporter constructs are formed by a minimal promoter containing binding sites for LexA or Gal4 respectively.

Both systems are induced by addition of the receptor activating ligands and the expression level of the reporter reflects transcription activation by the NR.

10 Binding of ligands to NRs can also be detected in competition-binding assays with purified NRs or NR LBDs. A specific radiolabeled ligand binds to NRs in the presence of unlabeled test compounds. The amount of receptor-bound radioactivity gives information about the affinity of the test compounds. In the scintillation proximity assay (SPA) biotinylated NR LBDs are linked to streptavidine coated beads impregnated with scintillant (Nichols et al., 1998). Radioactive counts 15 are observed only when the receptor-ligand complex is captured on the surface of the bead, eliminating the need to separate bound from free ligand. Therefore SPA is fully automatable and compatible to high throughput.

20 The homogenous fluorescence polarization-based ligand-binding assay utilizes His- and GST-tagged LBDs of steroid NR and a selective high affinity fluorescent-labeled ligand (Rosen et al., 2003). The binding affinity of a test compound is determined by displacement of the fluorescent steroid, accompanied by a change in the polarization value.

The competition assays are sensitive tools to identify NR ligands but cannot distinguish between agonists or antagonists, because only the affinity not the potential to induce conformational changes is measured.

25 The present invention also includes cell-free assays. Such assays involve contacting a form of PPARA (e.g., full-length PPARA, a biologically active fragment of PPARA, or a fusion protein comprising all or a portion of PPARA) with a test compound and determining the ability of the test compound to bind to PPARA. Binding of the test compound to PPARA can be determined either directly or indirectly as described above. In one embodiment, the assay includes contacting 30 PPARA with a known compound which binds PPARA to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with PPARA, wherein determining the ability of the test compound to interact with PPARA

comprises determining the ability of the test compound to preferentially bind to PPAR $\alpha$  as compared to the known compound.

The cell-free assays of the present invention are amenable to use of either a membrane-bound form of PPAR $\alpha$  or a soluble fragment thereof. In the case of cell-free assays comprising the membrane-bound form of the polypeptide, it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the polypeptide is maintained in solution. Examples of such solubilizing agents include but are not limited to non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton X-100, Triton X-114, Thesit, Isotridecy poly(ethylene glycol ether) $n$ , 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In various embodiments of the above assay methods of the present invention, it may be desirable to immobilize PPAR $\alpha$  (or a PPAR $\alpha$  target molecule) to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to PPAR $\alpha$ , or interaction of PPAR $\alpha$  with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and microcentrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase (GST) fusion proteins or glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or PPAR $\alpha$ , and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components and complex formation is measured either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity of PPAR $\alpha$  can be determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either PPAR $\alpha$  or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated polypeptide of the invention or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, Ill.), and immobilized in the

wells of streptavidin-coated plates (Pierce Chemical). Alternatively, antibodies reactive with PPARA or target molecules but which do not interfere with binding of the polypeptide of the invention to its target molecule can be derivatized to the wells of the plate, and unbound target or polypeptide of the invention trapped in the wells by antibody conjugation. Methods for detecting 5 such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with PPARA or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with PPARA or target molecule.

The screening assay can also involve monitoring the expression of PPARA. For example, 10 regulators of expression of PPARA can be identified in a method in which a cell is contacted with a candidate compound and the expression of PPARA protein or mRNA in the cell is determined. The level of expression of PPARA protein or mRNA the presence of the candidate compound is compared to the level of expression of PPARA protein or mRNA in the absence of the candidate compound. The candidate compound can then be identified as a regulator of expression of 15 PPARA based on this comparison. For example, when expression of PPARA protein or mRNA protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of PPARA protein or mRNA expression. Alternatively, when expression of PPARA protein or mRNA is less (statistically significantly less) in the presence of the candidate compound than in its absence, the 20 candidate compound is identified as an inhibitor of PPARA protein or mRNA expression. The level of PPARA protein or mRNA expression in the cells can be determined by methods described below.

#### *Binding Assays*

For binding assays, the test compound is preferably a small molecule which binds to and occupies 25 the active site of PPARA polypeptide, thereby making the ligand binding site inaccessible to substrate such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules. Potential ligands which bind to a polypeptide of the invention include, but are not limited to, the natural ligands of known PPARANuclear Receptors and analogues or derivatives thereof.

30 In binding assays, either the test compound or the PPARA polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horse-radish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to PPARA polypeptide can then be accomplished, for example, by direct counting of radio-emission, by scintillation counting, or by determining conversion of an appropriate substrate to a

detectable product. Alternatively, binding of a test compound to a PPARA polypeptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a PPARA polypeptide. A microphysiometer (e.g., Cytosensor<sup>TM</sup>) is an analytical instrument that measures the rate at which a cell acidifies its 5 environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and PPARA [Haseloff, (1988)].

Determining the ability of a test compound to bind to PPARA also can be accomplished using a 10 technology such as real-time Bimolecular Interaction Analysis (BIA) [McConnell, (1992); Sjolander, (1991)]. BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIACore<sup>TM</sup>). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, a PPARA-like polypeptide can be used as a “bait protein” in 15 a two-hybrid assay or three-hybrid assay [Szabo, (1995); U.S. 5,283,317], to identify other proteins which bind to or interact with PPARA and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist 20 of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding PPARA can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct a DNA sequence that encodes an unidentified protein (“prey” or “sample”) can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the “bait” and the “prey” proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are 25 brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein which interacts with PPARA.

30 It may be desirable to immobilize either the PPARA (or polynucleotide) or the test compound to facilitate separation of the bound form from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the PPARA-like polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes,

silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach PPARA-like polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide 5 (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to PPARA (or a polynucleotide encoding for PPARA) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

10 In one embodiment, PPARA is a fusion protein comprising a domain that allows binding of PPARA to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed PPARA; the mixture is then incubated under conditions conducive to complex 15 formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used 20 in the screening assays of the invention. For example, either PPARA (or a polynucleotide encoding PPARA) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated PPARA (or a polynucleotide encoding biotinylated PPARA) or test compounds can be prepared from biotin-NHS (N-hydroxysuccinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the 25 wells of streptavidin-coated plates (Pierce Chemical). Alternatively, antibodies which specifically bind to PPARA, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of PPARA, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to PPARA polypeptide or test compound, enzyme-linked assays which rely on detecting 30 an activity of PPARA polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a PPARA polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a PPARA polypeptide or polynucleotide

can be used in a cell-based assay system. A PPARA polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to PPARA or a polynucleotide encoding PPARA is determined as described above.

*Test Compounds*

5     Suitable test compounds for use in the screening assays of the invention can be obtained from any suitable source, e.g., conventional compound libraries. The test compounds can also be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the “one-bead one-compound” library method; 10 and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds [Lam, (1997)]. Examples of methods for the synthesis of molecular libraries can be found in the art. Libraries of compounds may be presented in solution or on beads, bacteria, spores, plasmids or phage.

15    *Modeling of Regulators*

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate PPARA expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be ligand binding sites, such as the interaction domain of the ligand 20 with PPARA. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

25    Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intramolecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with 30 a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing 5 molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

10 Finally, having determined the structure of the active site, either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer 15 assisted. These compounds found from this search are potential PPARA modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered 20 structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved specificity or activity.

### **Therapeutic Indications and Methods**

25 It was found by the present applicant that PPARA is expressed in various human tissues.

#### *Neurology*

CNS disorders include disorders of the central nervous system as well as disorders of the peripheral nervous system.

CNS disorders include, but are not limited to brain injuries, cerebrovascular diseases and their 30 consequences, Parkinson's disease, corticobasal degeneration, motor neuron disease, dementia, including ALS, multiple sclerosis, traumatic brain injury, stroke, post-stroke, post-traumatic brain injury, and small-vessel cerebrovascular disease. Dementias, such as Alzheimer's disease,

vascular dementia, dementia with Lewy bodies, frontotemporal dementia and Parkinsonism linked to chromosome 17, frontotemporal dementias, including Pick's disease, progressive nuclear palsy, corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jakob dementia, HIV dementia, schizophrenia with dementia, and Korsakoff's psychosis, within the 5 meaning of the definition are also considered to be CNS disorders.

Similarly, cognitive-related disorders, such as mild cognitive impairment, age-associated memory impairment, age-related cognitive decline, vascular cognitive impairment, attention deficit disorders, attention deficit hyperactivity disorders, and memory disturbances in children with learning disabilities are also considered to be CNS disorders.

10 Pain, within the meaning of this definition, is also considered to be a CNS disorder. Pain can be associated with CNS disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (e.g., infarct, hemorrhage, vascular malformation). Non-central neuropathic pain includes that associated with post mastectomy pain, phantom feeling, reflex 15 sympathetic dystrophy (RSD), trigeminal neuralgiaradioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (e.g., diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain associated with 20 peripheral nerve damage, central pain (i.e. due to cerebral ischemia) and various chronic pain i.e., lumbago, back pain (low back pain), inflammatory and/or rheumatic pain. Headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania are also CNS disorders.

25 Visceral pain such as pancreatitis, intestinal cystitis, dysmenorrhea, irritable Bowel syndrome, Crohn's disease, biliary colic, ureteral colic, myocardial infarction and pain syndromes of the pelvic cavity, e.g., vulvodynia, orchialgia, urethral syndrome and protatodynia are also CNS disorders.

30 Also considered to be a disorder of the nervous system are acute pain, for example postoperative pain, and pain after trauma.

The human PPARA is highly expressed in the following brain tissues: cerebellum (right), cerebellum (left), cerebral cortex, frontal lobe, Alzheimer brain frontal lobe, occipital lobe, temporal lobe, pons, substantia nigra, corpus callosum, hippocampus, thalamus, spinal cord,

neuroblastoma SH-SY5Y cells, retina. The expression in brain tissues and in particular the differential expression between diseased tissue Alzheimer brain frontal lobe and healthy tissue frontal lobe demonstrates that the human PPARA or mRNA can be utilized to diagnose nervous system diseases. Additionally the activity of the human PPARA can be modulated to treat nervous system diseases.

5

### *Cardiovascular Disorders*

Heart failure is defined as a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirement of the metabolizing tissue. It includes all forms of pumping failures such as high-output and low-output, acute and chronic, right-sided or left-sided, systolic or diastolic, independent of the underlying cause.

10 Myocardial infarction (MI) is generally caused by an abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by arteriosclerosis. MI prophylaxis (primary and secondary prevention) is included as well as the acute treatment of MI and the prevention of complications.

15 Ischemic diseases are conditions in which the coronary flow is restricted resulting in a perfusion which is inadequate to meet the myocardial requirement for oxygen. This group of diseases includes stable angina, unstable angina and asymptomatic ischemia.

Arrhythmias include all forms of atrial and ventricular tachyarrhythmias, atrial tachycardia, atrial 20 flutter, atrial fibrillation, atrio-ventricular reentrant tachycardia, preexcitation syndrome, ventricular tachycardia, ventricular flutter, ventricular fibrillation, as well as bradycardic forms of arrhythmias.

25 Hypertensive vascular diseases include primary as well as all kinds of secondary arterial hypertension, renal, endocrine, neurogenic, others. The genes may be used as drug targets for the treatment of hypertension as well as for the prevention of all complications arising from cardiovascular diseases.

Peripheral vascular diseases are defined as vascular diseases in which arterial and/or venous flow is reduced resulting in an imbalance between blood supply and tissue oxygen demand. It includes 30 chronic peripheral arterial occlusive disease (PAOD), acute arterial thrombosis and embolism, inflammatory vascular disorders, Raynaud's phenomenon and venous disorders.

Atherosclerosis is a cardiovascular disease in which the vessel wall is remodeled, compromising the lumen of the vessel. The atherosclerotic remodeling process involves accumulation of cells, both smooth muscle cells and monocyte/macrophage inflammatory cells, in the intima of the vessel wall. These cells take up lipid, likely from the circulation, to form a mature atherosclerotic lesion.

5 Although the formation of these lesions is a chronic process, occurring over decades of an adult human life, the majority of the morbidity associated with atherosclerosis occurs when a lesion ruptures, releasing thrombogenic debris that rapidly occludes the artery. When such an acute event occurs in the coronary artery, myocardial infarction can ensue, and in the worst case, can result in death.

10 The formation of the atherosclerotic lesion can be considered to occur in five overlapping stages such as migration, lipid accumulation, recruitment of inflammatory cells, proliferation of vascular smooth muscle cells, and extracellular matrix deposition. Each of these processes can be shown to occur in man and in animal models of atherosclerosis, but the relative contribution of each to the pathology and clinical significance of the lesion is unclear.

15 Thus, a need exists for therapeutic methods and agents to treat cardiovascular pathologies, such as atherosclerosis and other conditions related to coronary artery disease.

Cardiovascular diseases include but are not limited to disorders of the heart and the vascular system like congestive heart failure, myocardial infarction, ischemic diseases of the heart, all kinds of atrial and ventricular arrhythmias, hypertensive vascular diseases, peripheral vascular 20 diseases, and atherosclerosis.

Too high or too low levels of fats in the bloodstream, especially cholesterol, can cause long-term problems. The risk to develop atherosclerosis and coronary artery or carotid artery disease (and thus the risk of having a heart attack or stroke) increases with the total cholesterol level increasing. Nevertheless, extremely low cholesterol levels may not be healthy. Examples of disorders of lipid 25 metabolism are hyperlipidemia (abnormally high levels of fats (cholesterol, triglycerides, or both) in the blood, may be caused by family history of hyperlipidemia), obesity, a high-fat diet, lack of exercise, moderate to high alcohol consumption, cigarette smoking, poorly controlled diabetes, and an underactive thyroid gland), hereditary hyperlipidemias (type I hyperlipoproteinemia (familial hyperchylomicronemia), type II hyperlipoproteinemia (familial hypercholesterolemia), type III 30 hyperlipoproteinemia, type IV hyperlipoproteinemia, or type V hyperlipoproteinemia), hypolipoproteinemia, lipidoses (caused by abnormalities in the enzymes that metabolize fats), Gaucher's disease, Niemann-Pick disease, Fabry's disease, Wolman's disease, cerebrotendinous xanthomatosis, sitosterolemia, Refsum's disease, or Tay-Sachs disease.

Kidney disorders may lead to hypertension or hypotension. Examples for kidney problems possibly leading to hypertension are renal artery stenosis, pyelonephritis, glomerulonephritis, kidney tumors, polycistic kidney disease, injury to the kidney, or radiation therapy affecting the kidney. Excessive urination may lead to hypotension.

5 The human PPARA is highly expressed in the following cardiovascular related tissues: fetal heart, heart, pericardium, heart atrium (right), heart atrium (left), heart ventricle (left), heart ventricle (right), heart apex, Purkinje fibers, interventricular septum, pulmonic valve, fetal liver, liver, liver liver cirrhosis, liver tumor, fetal kidney, kidney, kidney tumor. Expression in the above mentioned tissues demonstrates that the human PPARA or mRNA can be utilized to diagnose of 10 cardiovascular diseases. Additionally the activity of the human PPARA can be modulated to treat cardiovascular diseases.

The human PPARA is highly expressed in liver tissues: fetal liver, liver, liver liver cirrhosis, liver tumor. Expression in liver tissues demonstrates that the human PPARA or mRNA can be utilized to diagnose of dyslipidemia disorders as an cardiovascular disorder. Additionally the activity of the 15 human PPARA can be modulated to treat - but not limited to - dyslipidemia disorders.

The human PPARA is highly expressed in kidney tissues : fetal kidney, kidney, kidney tumor. Expression in kidney tissues demonstrates that the human PPARA or mRNA can be utilized to diagnose of blood pressure disorders as an cardiovascular disorder. Additionally the activity of the human PPARA can be modulated to treat - but not limited to - blood pressure disorders as 20 hypertension or hypotension.

#### *Hematological Disorders*

Hematological disorders comprise diseases of the blood and all its constituents as well as diseases of organs and tissues involved in the generation or degradation of all the constituents of the blood. They include but are not limited to 1) Anemias, 2) Myeloproliferative Disorders, 3) Hemorrhagic 25 Disorders, 4) Leukopenia, 5) Eosinophilic Disorders, 6) Leukemias, 7) Lymphomas, 8) Plasma Cell Dyscrasias, 9) Disorders of the Spleen in the course of hematological disorders. Disorders according to 1) include, but are not limited to anemias due to defective or deficient hem synthesis, deficient erythropoiesis. Disorders according to 2) include, but are not limited to polycythemia vera, tumor-associated erythrocytosis, myelofibrosis, thrombocythemia. Disorders according to 3) 30 include, but are not limited to vasculitis, thrombocytopenia, heparin-induced thrombocytopenia, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, hereditary and acquired disorders of platelet function, hereditary coagulation disorders. Disorders according to 4) include, but are not limited to neutropenia, lymphocytopenia. Disorders according to 5) include, but are not

limited to hypereosinophilia, idiopathic hypereosinophilic syndrome. Disorders according to 6) include, but are not limited to acute myeloic leukemia, acute lymphoblastic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome. Disorders according to 7) include, but are not limited to Hodgkin's disease, non-Hodgkin's lymphoma, 5 Burkitt's lymphoma, mycosis fungoides cutaneous T-cell lymphoma. Disorders according to 8) include, but are not limited to multiple myeloma, macroglobulinemia, heavy chain diseases. In extension of the preceding idiopathic thrombocytopenic purpura, iron deficiency anemia, megaloblastic anemia (vitamin B12 deficiency), aplastic anemia, thalassemia, malignant lymphoma bone marrow invasion, malignant lymphoma skin invasion, hemolytic uremic 10 syndrome, giant platelet disease are considered to be hematological diseases too.

The human PPARA is highly expressed in the following tissues of the hematological system: bone marrow stromal cells, cord blood CD34+ cells, neutrophils cord blood, neutrophils peripheral blood. The expression in the above mentioned tissues demonstrates that the human PPARA or mRNA can be utilized to diagnose of hematological diseases. Additionally the activity of the 15 human PPARA can be modulated to treat hematological disorders.

#### *Gastrointestinal and Liver Diseases*

Gastrointestinal diseases comprise primary or secondary, acute or chronic diseases of the organs of the gastrointestinal tract which may be acquired or inherited, benign or malignant or metaplastic, and which may affect the organs of the gastrointestinal tract or the body as a whole. They comprise 20 but are not limited to 1) disorders of the esophagus like achalasia, vigorous achalasia, dysphagia, cricopharyngeal incoordination, pre-esophageal dysphagia, diffuse esophageal spasm, globus sensation, Barrett's metaplasia, gastroesophageal reflux, 2) disorders of the stomach and duodenum like functional dyspepsia, inflammation of the gastric mucosa, gastritis, stress gastritis, chronic erosive gastritis, atrophy of gastric glands, metaplasia of gastric tissues, gastric ulcers, 25 duodenal ulcers, neoplasms of the stomach, 3) disorders of the pancreas like acute or chronic pancreatitis, insufficiency of the exocrine or endocrine tissues of the pancreas like steatorrhea, diabetes, neoplasms of the exocrine or endocrine pancreas like 3.1) multiple endocrine neoplasia syndrome, ductal adenocarcinoma, cystadenocarcinoma, islet cell tumors, insulinoma, gastrinoma, carcinoid tumors, glucagonoma, Zollinger-Ellison syndrome, Vipoma syndrome, malabsorption 30 syndrome, 4) disorders of the bowel like chronic inflammatory diseases of the bowel, Crohn's disease, ileus, diarrhea and constipation, colonic inertia, megacolon, malabsorption syndrome, ulcerative colitis, 4.1) functional bowel disorders like irritable bowel syndrome, 4.2) neoplasms of the bowel like familial polyposis, adenocarcinoma, primary malignant lymphoma, carcinoid tumors, Kaposi's sarcoma, polyps, cancer of the colon and rectum.

Liver diseases comprise primary or secondary, acute or chronic diseases or injury of the liver which may be acquired or inherited, benign or malignant, and which may affect the liver or the body as a whole. They comprise but are not limited to disorders of the bilirubin metabolism, jaundice, syndroms of Gilbert's, Crigler-Najjar, Dubin-Johnson and Rotor; intrahepatic cholestasis, 5 hepatomegaly, portal hypertension, ascites, Budd-Chiari syndrome, portal-systemic encephalopathy, fatty liver, steatosis, Reye's syndrome, liver diseases due to alcohol, alcoholic hepatitis or cirrhosis, fibrosis and cirrhosis, fibrosis and cirrhosis of the liver due to inborn errors of metabolism or exogenous substances, storage diseases, syndromes of Gaucher's, Zellweger's, Wilson's - disease, acute or chronic hepatitis, viral hepatitis and its variants, inflammatory conditions of the liver due 10 to viruses, bacteria, fungi, protozoa, helminths; drug induced disorders of the liver, chronic liver diseases like primary sclerosing cholangitis, alpha<sub>1</sub>-antitrypsin-deficiency, primary biliary cirrhosis, postoperative liver disorders like postoperative intrahepatic cholestasis, hepatic granulomas, vascular liver disorders associated with systemic disease, benign or malignant neoplasms of the liver, disturbance of liver metabolism in the new-born or prematurely born.

15 The human PPAR $\alpha$  is highly expressed in the following tissues of the gastroenterological system: stomach, stomach tumor, small intestine, ileum, ileum tumor, fetal liver, liver, liver liver cirrhosis, liver tumor, HEP G2 cells. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue liver liver cirrhosis and healthy tissue liver demonstrates that the human PPAR $\alpha$  or mRNA can be utilized to diagnose of gastroenterological 20 disorders. Additionally the activity of the human PPAR $\alpha$  can be modulated to treat gastroenterological disorders.

#### *Cancer Disorders*

Cancer disorders within the scope of this definition comprise any disease of an organ or tissue in mammals characterized by poorly controlled or uncontrolled multiplication of normal or abnormal 25 cells in that tissue and its effect on the body as a whole. Cancer diseases within the scope of the definition comprise benign neoplasms, dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations like e.g. leukoplakias which often precede a breakout of cancer. Cells and tissues are cancerous when they grow more rapidly than normal 30 cells, displacing or spreading into the surrounding healthy tissue or any other tissues of the body described as metastatic growth, assume abnormal shapes and sizes, show changes in their nucleocytoplasmatic ratio, nuclear polychromasia, and finally may cease. Cancerous cells and tissues may affect the body as a whole when causing paraneoplastic syndromes or if cancer occurs within a vital organ or tissue, normal function will be impaired or halted, with possible fatal results. The ultimate involvement of a vital organ by cancer, either primary or metastatic, may lead

to the death of the mammal affected. Cancer tends to spread, and the extent of its spread is usually related to an individual's chances of surviving the disease. Cancers are generally said to be in one of three stages of growth: early, or localized, when a tumor is still confined to the tissue of origin, or primary site; direct extension, where cancer cells from the tumour have invaded adjacent tissue 5 or have spread only to regional lymph nodes; or metastasis, in which cancer cells have migrated to distant parts of the body from the primary site, via the blood or lymph systems, and have established secondary sites of infection. Cancer is said to be malignant because of its tendency to cause death if not treated. Benign tumors usually do not cause death, although they may if they interfere with a normal body function by virtue of their location, size, or paraneoplastic side 10 effects. Hence benign tumors fall under the definition of cancer within the scope of this definition as well. In general, cancer cells divide at a higher rate than do normal cells, but the distinction between the growth of cancerous and normal tissues is not so much the rapidity of cell division in the former as it is the partial or complete loss of growth restraint in cancer cells and their failure to differentiate into a useful, limited tissue of the type that characterizes the functional equilibrium of 15 growth of normal tissue. Cancer tissues may express certain molecular receptors and probably are influenced by the host's susceptibility and immunity and it is known that certain cancers of the breast and prostate, for example, are considered dependent on specific hormones for their existence. The term "cancer" under the scope of the definition is not limited to simple benign neoplasia but comprises any other benign and malign neoplasia like 1) Carcinoma, 2) Sarcoma, 3) 20 Carcinosarcoma, 4) Cancers of the blood-forming tissues, 5) tumors of nerve tissues including the brain, 6) cancer of skin cells. Cancer according to 1) occurs in epithelial tissues, which cover the outer body (the skin) and line mucous membranes and the inner cavitary structures of organs e.g. such as the breast, lung, the respiratory and gastrointestinal tracts, the endocrine glands, and the genitourinary system. Ductal or glandular elements may persist in epithelial tumors, as in 25 adenocarcinomas like e.g. thyroid adenocarcinoma, gastric adenocarcinoma, uterine adenocarcinoma. Cancers of the pavement-cell epithelium of the skin and of certain mucous membranes, such as e.g. cancers of the tongue, lip, larynx, urinary bladder, uterine cervix, or penis, may be termed epidermoid or squamous-cell carcinomas of the respective tissues and are in the scope of the definition of cancer as well. Cancer according to 2) develops in connective tissues, including 30 fibrous tissues, adipose (fat) tissues, muscle, blood vessels, bone, and cartilage like e.g. osteogenic sarcoma; liposarcoma, fibrosarcoma, synovial sarcoma. Cancer according to 3) is cancer that develops in both epithelial and connective tissue. Cancer disease within the scope of this definition may be primary or secondary, whereby primary indicates that the cancer originated in the tissue where it is found rather than was established as a secondary site through metastasis from another 35 lesion. Cancers and tumor diseases within the scope of this definition may be benign or malign and may affect all anatomical structures of the body of a mammal. By example but not limited to they

comprise cancers and tumor diseases of I) the bone marrow and bone marrow derived cells (leukemias), II) the endocrine and exocrine glands like e.g. thyroid, parathyroid, pituitary, adrenal glands, salivary glands, pancreas III) the breast, like e.g. benign or malignant tumors in the mammary glands of either a male or a female, the mammary ducts, adenocarcinoma, medullary carcinoma, comedo carcinoma, Paget's disease of the nipple, inflammatory carcinoma of the young woman, IV) the lung, V) the stomach, VI) the liver and spleen, VII) the small intestine, VIII) the colon, IX) the bone and its supportive and connective tissues like malignant or benign bone tumour, e.g. malignant osteogenic sarcoma, benign osteoma, cartilage tumors; like malignant chondrosarcoma or benign chondroma; bone marrow tumors like malignant myeloma or benign 5 eosinophilic granuloma, as well as metastatic tumors from bone tissues at other locations of the body; X) the mouth, throat, larynx, and the esophagus, XI) the urinary bladder and the internal and external organs and structures of the urogenital system of male and female like ovaries, uterus, cervix of the uterus, testes, and prostate gland, XII) the prostate, XIII) the pancreas, like ductal 10 carcinoma of the pancreas; XIV) the lymphatic tissue like lymphomas and other tumors of lymphoid origin, XV) the skin, XVI) cancers and tumor diseases of all anatomical structures belonging to the respiration and respiratory systems including thoracal muscles and linings, XVII) 15 primary or secondary cancer of the lymph nodes XVIII) the tongue and of the bony structures of the hard palate or sinuses, XVIV) the mouth, cheeks, neck and salivary glands, XX) the blood vessels including the heart and their linings, XXI) the smooth or skeletal muscles and their 20 ligaments and linings, XXII) the peripheral, the autonomous, the central nervous system including the cerebellum, XXIII) the adipose tissue.

The human PPAR $\alpha$  is highly expressed in the following cancer tissues: stomach tumor, ileum tumor, liver tumor, HEP G2 cells, ovary tumor, breast tumor, kidney tumor. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue 25 stomach tumor and healthy tissue stomach, between diseased tissue ileum tumor and healthy tissue ileum, between diseased tissue liver tumor and healthy tissue liver, between diseased tissue HEP G2 cells and healthy tissue liver, between diseased tissue ovary tumor and healthy tissue ovary, between diseased tissue breast tumor and healthy tissue breast, between diseased tissue kidney tumor and healthy tissue kidney demonstrates that the human PPAR $\alpha$  or mRNA can be utilized to 30 diagnose of cancer. Additionally the activity of the human PPAR $\alpha$  can be modulated to treat cancer.

#### *Inflammatory Diseases*

Inflammatory diseases comprise diseases triggered by cellular or non-cellular mediators of the immune system or tissues causing the inflammation of body tissues and subsequently producing an

acute or chronic inflammatory condition. Examples for such inflammatory diseases are hypersensitivity reactions of type I – IV, for example but not limited to hypersensitivity diseases of the lung including asthma, atopic diseases, allergic rhinitis or conjunctivitis, angioedema of the lids, hereditary angioedema, antireceptor hypersensitivity reactions and autoimmune diseases, 5 Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, pemphigus, myasthenia gravis, Grave's and Raynaud's disease, type B insulin-resistant diabetes, rheumatoid arthritis, psoriasis, Crohn's disease, scleroderma, mixed connective tissue disease, polymyositis, sarcoidosis, glomerulonephritis, acute or chronic host versus graft reactions.

The human PPAR $\alpha$  is highly expressed in the following tissues of the immune system and tissues 10 responsive to components of the immune system as well as in the following tissues responsive to mediators of inflammation: liver liver cirrhosis, neutrophils cord blood, neutrophils peripheral blood. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue liver liver cirrhosis and healthy tissue liver demonstrates that the human 15 PPAR $\alpha$  or mRNA can be utilized to diagnose of inflammatory diseases. Additionally the activity of the human PPAR $\alpha$  can be modulated to treat inflammatory diseases.

#### *Disorders Related to Pulmology*

Asthma is thought to arise as a result of interactions between multiple genetic and environmental factors and is characterized by three major features: 1) intermittent and reversible airway 20 obstruction caused by bronchoconstriction, increased mucus production, and thickening of the walls of the airways that leads to a narrowing of the airways, 2) airway hyperresponsiveness, and 3) airway inflammation. Certain cells are critical to the inflammatory reaction of asthma and they include T cells and antigen presenting cells, B cells that produce IgE, and mast cells, basophils, eosinophils, and other cells that bind IgE. These effector cells accumulate at the site of allergic 25 reaction in the airways and release toxic products that contribute to the acute pathology and eventually to tissue destruction related to the disorder. Other resident cells, such as smooth muscle cells, lung epithelial cells, mucus-producing cells, and nerve cells may also be abnormal in individuals with asthma and may contribute to its pathology. While the airway obstruction of asthma, presenting clinically as an intermittent wheeze and shortness of breath, is generally the most pressing symptom of the disease requiring immediate treatment, the inflammation and tissue 30 destruction associated with the disease can lead to irreversible changes that eventually make asthma a chronic and disabling disorder requiring long-term management.

Chronic obstructive pulmonary (or airways) disease (COPD) is a condition defined physiologically as airflow obstruction that generally results from a mixture of emphysema and peripheral airway obstruction due to chronic bronchitis [Botstein, 1980]. Emphysema is characterised by destruction

of alveolar walls leading to abnormal enlargement of the air spaces of the lung. Chronic bronchitis is defined clinically as the presence of chronic productive cough for three months in each of two successive years. In COPD, airflow obstruction is usually progressive and is only partially reversible. By far the most important risk factor for development of COPD is cigarette smoking,  
5 although the disease does also occur in non-smokers.

The human PPAR $\alpha$  is highly expressed in the following tissues of the respiratory system: neutrophils cord blood, neutrophils peripheral blood. The expression in the above mentioned tissues demonstrates that the human PPAR $\alpha$  or mRNA can be utilized to diagnose of respiratory diseases. Additionally the activity of the human PPAR $\alpha$  can be modulated to treat those diseases.

10 *Disorders Related to Urology*

Genitourinary disorders comprise benign and malign disorders of the organs constituting the genitourinary system of female and male, renal diseases like acute or chronic renal failure, immunologically mediated renal diseases like renal transplant rejection, lupus nephritis, immune complex renal diseases, glomerulopathies, nephritis, toxic nephropathy, obstructive uropathies like  
15 benign prostatic hyperplasia (BPH), neurogenic bladder syndrome, urinary incontinence like urge-, stress-, or overflow incontinence, pelvic pain; and erectile dysfunction.

The human PPAR $\alpha$  is highly expressed in the following urological tissues: spinal cord, ureter, fetal kidney, kidney, kidney tumor. The expression in the above mentioned tissues demonstrates that the human PPAR $\alpha$  or mRNA can be utilized to diagnose of urological disorders. Additionally  
20 the activity of the human PPAR $\alpha$  can be modulated to treat urological disorders.

The human PPAR $\alpha$  is highly expressed in spinal cord tissues: spinal cord. Expression in spinal cord tissues demonstrates that the human PPAR $\alpha$  or mRNA can be utilized to diagnose of incontinence as an urological disorder. The spinal cord tissues are involved in the neuronal regulation of the urological system. Additionally the activity of the human PPAR $\alpha$  can be  
25 modulated to treat - but not limited to - incontinence.

*Applications*

The present invention provides for both prophylactic and therapeutic methods for cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

30 The regulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of PPAR $\alpha$ . An agent that modulates activity can be an agent as described

herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of the polypeptide, a peptide, a peptidomimetic, or any small molecule. In one embodiment, the agent stimulates one or more of the biological activities of PPARA. Examples of such stimulatory agents include the active PPARA and nucleic acid molecules encoding a portion of PPARA. In another embodiment, 5 the agent inhibits one or more of the biological activities of PPARA. Examples of such inhibitory agents include antisense nucleic acid molecules and antibodies. These regulatory methods can be performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by unwanted expression or activity 10 of PPARA or a protein in the PPARA signaling pathway. In one embodiment, the method involves administering an agent like any agent identified or being identifiable by a screening assay as described herein, or combination of such agents that modulate say upregulate or downregulate the expression or activity of PPARA or of any protein in the PPARA signaling pathway. In another embodiment, the method involves administering a regulator of PPARA as therapy to compensate 15 for reduced or undesirably low expression or activity of PPARA or a protein in the PPARA signaling pathway.

Stimulation of activity or expression of PPARA is desirable in situations in which activity or expression is abnormally low and in which increased activity is likely to have a beneficial effect. Conversely, inhibition of activity or expression of PPARA is desirable in situations in which 20 activity or expression of PPARA is abnormally high and in which decreasing its activity is likely to have a beneficial effect.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

## 25 **Pharmaceutical Compositions**

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

The nucleic acid molecules, polypeptides, and antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for 30 administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like,

compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

5 The invention includes pharmaceutical compositions comprising a regulator of PPARA expression or activity (and/or a regulator of the activity or expression of a protein in the PPARA signaling pathway) as well as methods for preparing such compositions by combining one or more such regulators and a pharmaceutically acceptable carrier. Also within the invention are pharmaceutical compositions comprising a regulator identified using the screening assays of the invention  
10 packaged with instructions for use. For regulators that are antagonists of PPARA activity or which reduce PPARA expression, the instructions would specify use of the pharmaceutical composition for treatment of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases. For regulators that are agonists of PPARA activity or increase PPARA expression, the instructions  
15 would specify use of the pharmaceutical composition for treatment of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

An antagonist of PPARA may be produced using methods which are generally known in the art. In particular, purified PPARA may be used to produce antibodies or to screen libraries of  
20 pharmaceutical agents to identify those which specifically bind PPARA. Antibodies to PPARA may also be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain antibodies, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies like those which inhibit dimer formation are especially preferred for therapeutic use.

25 In another embodiment of the invention, the polynucleotides encoding PPARA, or any fragment or complement thereof, may be used for therapeutic purposes. In one aspect, the complement of the polynucleotide encoding PPARA may be used in situations in which it would be desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding PPARA. Thus, complementary molecules or  
30 fragments may be used to modulate PPARA activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligonucleotides or larger fragments can be designed from various locations along the coding or control regions of sequences encoding PPARA.

Expression vectors derived from retroviruses, adenoviruses, or herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of nucleotide sequences to the targeted organ, tissue, or cell population. Methods which are well known to those skilled in the art can be used to construct vectors which will express nucleic acid sequence complementary to the polynucleotides 5 of the gene encoding PPARA. These techniques are described, for example, in [Scott and Smith (1990) *Science* 249:386-390].

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

10 An additional embodiment of the invention relates to the administration of a pharmaceutical composition containing PPARA in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of PPARA, antibodies to PPARA, and mimetics, agonists, antagonists, or inhibitors of PPARA. The compositions may be administered alone or in combination with at least one other agent, such as a 15 stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, 20 intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as 25 ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

30 Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EM<sup>TM</sup> (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to

the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, a pharmaceutically acceptable polyol like glycerol, propylene glycol, liquid polyethylene glycol, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin. Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a polypeptide or antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.

Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressurized container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or 5 transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or 10 creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the 15 compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza 20 Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for 25 ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active 30 compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. For pharmaceutical compositions which include an antagonist of

PPARA activity, a compound which reduces expression of PPARA, or a compound which reduces expression or activity of a protein in the PPARA signaling pathway or any combination thereof, the instructions for administration will specify use of the composition for cardiovascular diseases, 5 gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases. For pharmaceutical compositions which include an agonist of PPARA activity, a compound which increases expression of PPARA, or a compound which increases expression or activity of a protein in the PPARA signaling pathway or any combination thereof, the instructions for administration will specify use of the composition for cardiovascular diseases, 10 gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

### **Diagnostics**

In another embodiment, antibodies which specifically bind PPARA may be used for the diagnosis of disorders characterized by the expression of PPARA, or in assays to monitor patients being treated with PPARA or agonists, antagonists, and inhibitors of PPARA. Antibodies useful for 15 diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for PPARA include methods which utilize the antibody and a label to detect PPARA in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by covalent or non-covalent joining with a reporter molecule. A wide variety of reporter molecules, several of which are described above, are 20 known in the art and may be used.

A variety of protocols for measuring PPARA, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of PPARA expression. Normal or standard values for PPARA expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to PPARA 25 under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, preferably by photometric means. Quantities of PPARA expressed in subject samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding PPARA may be used for 30 diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of PPARA may be correlated with disease. The diagnostic assay may be used to distinguish between absence,

presence, and excess expression of PPARA, and to monitor regulation of PPARA levels during therapeutic intervention.

Polynucleotide sequences encoding PPARA may be used for the diagnosis of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases associated with expression of PPARA.

5 The polynucleotide sequences encoding PPARA may be used in Southern, Northern, or dot-blot analysis, or other membrane-based technologies; in PCR technologies; in dipstick, pin, and ELISA assays; and in microarrays utilizing fluids or tissues from patient biopsies to detect altered PPARA expression. Such qualitative or quantitative methods are well known in the art.

10 In a particular aspect, the nucleotide sequences encoding PPARA may be useful in assays that detect the presence of associated disorders, particularly those mentioned above. The nucleotide sequences encoding PPARA may be labelled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and

15 compared with a standard value. If the amount of signal in the patient sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding PPARA in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal

20 studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases associated with expression of PPARA, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, 25 encoding PPARA, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from 30 samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, large numbers of different small test compounds

are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with PPARA, or fragments thereof, and washed. Bound PPARA is then detected by methods well known in the art. Purified PPARA can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing 5 antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding PPARA specifically compete with a testcompound for binding PPARA. In this manner, antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with PPARA.

10 Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with PPARA, or fragments thereof, and washed. Bound PPARA is then 15 detected by methods well known in the art. Purified PPARA can also be coated directly onto plates for use in the afore-mentioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

20 In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding PPARA specifically compete with a testcompound for binding PPARA. In this manner, antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with PPARA.

### **Determination of a Therapeutically Effective Dose**

The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which 25 increases or decreases PPARA activity relative to PPARA activity which occurs in the absence of the therapeutically effective dose. For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes 30 for administration in humans.

Therapeutic efficacy and toxicity, e.g., ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population), can be determined by standard

pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD<sub>50</sub>/ED<sub>50</sub>. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for 5 human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration. The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient 10 levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life 15 and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 micrograms to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their 20 inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc. If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intra- 25 cellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun", and DEAE- or calcium phosphate-mediated transfection.

If the expression product is mRNA, the reagent is preferably an antisense oligonucleotide or a ribozyme. Polynucleotides which express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above. Preferably, a reagent reduces 30 expression of PPARA gene or the activity of PPARA by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of PPARA gene or the activity of PPARA can be assessed using methods well known in the art, such as hybridization of nucleotide probes to PPARA-specific mRNA, quantitative RT-PCR, immunologic detection of PPARA, or 35 measurement of PPARA activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects. Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, 10 humans.

Nucleic acid molecules of the invention are those nucleic acid molecules which are contained in a group of nucleic acid molecules consisting of (i) nucleic acid molecules encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, (ii) nucleic acid molecules comprising the sequence of SEQ ID NO: 1, (iii) nucleic acid molecules having the sequence of SEQ ID NO: 1, 15 (iv) nucleic acid molecules the complementary strand of which hybridizes under stringent conditions to a nucleic acid molecule of (i), (ii), or (iii); and (v) nucleic acid molecules the sequence of which differs from the sequence of a nucleic acid molecule of (iii) due to the degeneracy of the genetic code, wherein the polypeptide encoded by said nucleic acid molecule has PPARA activity.

20 Polypeptides of the invention are those polypeptides which are contained in a group of polypeptides consisting of (i) polypeptides having the sequence of SEQ ID NO: 2, (ii) polypeptides comprising the sequence of SEQ ID NO: 2, (iii) polypeptides encoded by nucleic acid molecules of the invention and (iv) polypeptides which show at least 99%, 98%, 95%, 90%, or 80% homology with a polypeptide of (i), (ii), or (iii), wherein said purified polypeptide has PPARA activity.

25 An object of the invention is a method of screening for therapeutic agents useful in the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising the steps of (i) contacting a test compound with a PPARA polypeptide, (ii) detect binding of said test compound to said PPARA 30 polypeptide. E.g., compounds that bind to the PPARA polypeptide are identified potential therapeutic agents for such a disease.

Another object of the invention is a method of screening for therapeutic agents useful in the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases,

neurological diseases and urological diseases in a mammal comprising the steps of (i) determining the activity of a PPAR $\alpha$  polypeptide at a certain concentration of a test compound or in the absence of said test compound, (ii) determining the activity of said polypeptide at a different concentration of said test compound. E.g., compounds that lead to a difference in the activity of the PPAR $\alpha$  polypeptide in (i) and (ii) are identified potential therapeutic agents for such a disease.

Another object of the invention is a method of screening for therapeutic agents useful in the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising the steps of (i) determining the activity of a PPAR $\alpha$  polypeptide at a certain concentration of a test compound, (ii) determining the activity of a PPAR $\alpha$  polypeptide at the presence of a compound known to be a regulator of a PPAR $\alpha$  polypeptide. E.g., compounds that show similar effects on the activity of the PPAR $\alpha$  polypeptide in (i) as compared to compounds used in (ii) are identified potential therapeutic agents for such a disease.

15 Other objects of the invention are methods of the above, wherein the step of contacting is in or at the surface of a cell.

Other objects of the invention are methods of the above, wherein the cell is in vitro.

Other objects of the invention are methods of the above, wherein the step of contacting is in a cell-free system.

20 Other objects of the invention are methods of the above, wherein the polypeptide is coupled to a detectable label.

Other objects of the invention are methods of the above, wherein the compound is coupled to a detectable label.

25 Other objects of the invention are methods of the above, wherein the test compound displaces a ligand which is first bound to the polypeptide.

Other objects of the invention are methods of the above, wherein the polypeptide is attached to a solid support.

Other objects of the invention are methods of the above, wherein the compound is attached to a solid support.

Another object of the invention is a method of screening for therapeutic agents useful in the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising the steps of (i) contacting a 5 test compound with a PPARA polynucleotide, (ii) detect binding of said test compound to said PPARA polynucleotide. Compounds that, e.g., bind to the PPARA polynucleotide are potential therapeutic agents for the treatment of such diseases.

Another object of the invention is the method of the above, wherein the nucleic acid molecule is RNA.

10 Another object of the invention is a method of the above, wherein the contacting step is in or at the surface of a cell.

Another object of the invention is a method of the above, wherein the contacting step is in a cell-free system.

15 Another object of the invention is a method of the above, wherein the polynucleotide is coupled to a detectable label.

Another object of the invention is a method of the above, wherein the test compound is coupled to a detectable label.

Another object of the invention is a method of diagnosing a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, 20 hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising the steps of (i) determining the amount of a PPARA polynucleotide in a sample taken from said mammal, (ii) determining the amount of PPARA polynucleotide in healthy and/or diseased mammal. A disease is diagnosed, e.g., if there is a substantial similarity in the amount of PPARA polynucleotide in said test mammal as compared to a diseased mammal.

25 Another object of the invention is a pharmaceutical composition for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a therapeutic agent which binds to a PPARA polypeptide.

30 Another object of the invention is a pharmaceutical composition for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological

diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a therapeutic agent which regulates the activity of a PPARA polypeptide.

Another object of the invention is a pharmaceutical composition for the treatment of a disease

5 comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a therapeutic agent which regulates the activity of a PPARA polypeptide, wherein said therapeutic agent is (i) a small molecule, (ii) an RNA molecule, (iii) an antisense oligonucleotide, (iv) a polypeptide, (v) an antibody, or (vi) a ribozyme.

10 Another object of the invention is a pharmaceutical composition for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a PPARA polynucleotide.

15 Another object of the invention is a pharmaceutical composition for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising a PPARA polypeptide.

20 Another object of the invention is the use of regulators of a PPARA for the preparation of a pharmaceutical composition for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal.

25 Another object of the invention is a method for the preparation of a pharmaceutical composition useful for the treatment of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal comprising the steps of (i) identifying a regulator of PPARA, (ii) determining whether said regulator ameliorates the symptoms of a disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases in a mammal; and (iii) combining of said regulator 30 with an acceptable pharmaceutical carrier.

Another object of the invention is the use of a regulator of PPARA for the regulation of PPARA activity in a mammal having a disease comprised in a group of diseases consisting of cardio-

vascular diseases, gastroenterological diseases, cancer, inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

The uses, methods or compositions of the invention are useful for each single disease comprised in a group of diseases consisting of cardiovascular diseases, gastroenterological diseases, cancer, 5 inflammation, hematological diseases, respiratory diseases, neurological diseases and urological diseases.

The expression of human PPARA in hematological and urological related tissues (as described above) suggests a particular – but not limited to – utilization of PPARA for diagnosis and modulation of hematological diseases and urological diseases. Furthermore the above described 10 expression suggest a – but not limited to – utilization of PPARA to cardiovascular diseases, infections, cancer, endocrinological diseases, metabolic diseases, gastroenterological diseases, respiratory diseases, inflammation, muscle-skeleton diseases and neurological diseases.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

**Examples****Example 1: Search for homologous sequences in public sequence data bases**

The degree of homology can readily be calculated by known methods. Preferred methods to determine homology are designed to give the largest match between the sequences tested.

5 Methods to determine homology are codified in publicly available computer programs such as BestFit, BLASTP, BLASTN, and FASTA. The BLAST programs are publicly available from NCBI and other sources in the internet.

For PPARA the following hits to known sequences were identified by using the BLAST algorithm [Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ; Nucleic Acids Res 1997 Sep 1; 25(17): 3389-402] and the following set of parameters: matrix = BLOSUM62 and low complexity filter. The following databases were searched: NCBI (non-redundant database) and DERWENT patent database (Geneseq).

The following hits were found:

>ref|NM\_005036.2| Homo sapiens peroxisome proliferative activated receptor, alpha(PPARA),

15 mRNA

Length = 1850, Score = 3557 bits (1850), Expect = 0.0, Identities = 1850/1850 (100%)

>gb|L02932.1|HUMPPAR Human peroxisome proliferator activated receptor mRNA, complete cds

Length = 1854, Score = 3505 bits (1823), Expect = 0.0, Identities = 1847/1854 (99%), Gaps =

20 4/1854 (0%)

>gb|S74349.1|S74349 hPPAR alpha =peroxisome proliferator activated receptor alpha

[human,liver, mRNA, 1731 nt]

Length = 1731, Score = 3328 bits (1731), Expect = 0.0, Identities = 1731/1731 (100%)

25

>emb|Y07619.1|HSPPARAGE H.sapiens mRNA for peroxisome proliferator-activated receptor alpha

Length = 1407, Score = 2682 bits (1395), Expect = 0.0, Identities = 1403/1407 (99%)

30

>gb|AF350327.1|AF350327 Canis familiaris peroxisome proliferator activated receptor alphanRNA, complete cds

Length = 2032, Score = 1698 bits (883), Expect = 0.0, Identities = 1245/1426 (87%)

>gb|AF229356.1|AF229356 Bos taurus peroxisome proliferator activated receptor alpha(PPARalpha) mRNA, partial cds

Length = 1411, Score = 1567 bits (815), Expect = 0.0, Identities = 1211/1409 (85%)

5 >dbj|AK081709.1| Mus musculus 16 days embryo head cDNA, RIKEN full-length enrichedlibrary, clone:C130070E08 product:peroxisome proliferatoractivated receptor alpha, full insert sequence Length = 2519, Score = 1534 bits (798), Expect = 0.0, Identities = 1204/1407 (85%)

10 >dbj|AK035676.1| Mus musculus adult male urinary bladder cDNA, RIKEN full-lengthenriched library, clone:9530085L04 product:peroxisomeproliferator activated receptor alpha, full insertsequence Length = 3413, Score = 1534 bits (798), Expect = 0.0, Identities = 1204/1407 (85%)

15 >ref|NM\_011144.2| Mus musculus peroxisome proliferator activated receptor alpha(Ppara), mRNA Length = 3413, Score = 1534 bits (798), Expect = 0.0, Identities = 1204/1407 (85%)

20 >emb|AJ555631.1|MAU555631 Mesocricetus auratus mRNA for peroxisome proliferator activatedreceptor alpha (ppara gene) Length = 1407, Score = 1529 bits (795), Expect = 0.0, Identities = 1203/1407 (85%)

>emb|X57638.1|MMPPAR Mouse mRNA for peroxisome proliferator activated receptor Length = 2081, Score = 1523 bits (792), Expect = 0.0, Identities = 1202/1407 (85%)

25 >ref|NM\_013196.1| Rattus norvegicus peroxisome proliferator activated receptor alpha(Ppara), mRNA Length = 2022, Score = 1506 bits (783), Expect = 0.0, Identities = 1199/1407 (85%)

30 >gb|M88592.1|RATPPAR Rattus rattus peroxisome proliferator activated receptor (PPAR) mRNA,complete cds Length = 2022, Score = 1506 bits (783), Expect = 0.0, Identities = 1199/1407 (85%)

>gb|AF228696.1|AF228696 Sus scrofa peroxisome proliferator activated receptor alpha(PPARalpha) mRNA, partial cds

35 Length = 1404, Score = 1475 bits (767), Expect = 0.0, Identities = 1191/1404 (84%)

>emb|AJ000222.1|CPAJ222 Cavia porcellus mRNA for peroxisome proliferator-activated receptoralpha

Length = 2073, Score = 1140 bits (593), Expect = 0.0, Identities = 1138/1408 (80%), Gaps = 3/1408 (0%)

5

>emb|AJ006218.1|CPO6218 Cavia porcellus mRNA for peroxisome proliferator-activated receptoralpha

Length = 1610, Score = 1098 bits (571), Expect = 0.0, Identities = 1132/1405 (80%), Gaps = 5/1405 (0%)

10

>gb|AF470455.1| Gallus gallus breed AA peroxisome proliferator-activated receptoralpha mRNA, complete cds

Length = 2100, Score = 1019 bits (530), Expect = 0.0, Identities = 1112/1403 (79%)

15

>gb|AF163809.1|AF163809 Gallus gallus peroxisome proliferator-activated receptor alpha(PPARalpha) mRNA, complete cds

Length = 2100, Score = 1019 bits (530), Expect = 0.0, Identities = 1112/1403 (79%)

20

>gb|AF463455.1| Phascolarctos cinereus peroxisome proliferator activated receptoralpha protein mRNA, complete cds

Length = 1515, Score = 990 bits (515), Expect = 0.0, Identities = 1111/1409 (78%)

>gb|AY170844.1| Mesocricetus auratus peroxisome proliferator activated receptor alpha(PPARalpha) mRNA, partial cds

25

Length = 831, Score = 987 bits (513), Expect = 0.0, Identities = 725/831 (87%)

>gb|AF481797.1| Anser anser peroxisome proliferator-activated receptor alpha(PPARalpha) mRNA, complete cds

Length = 1455, Score = 946 bits (492), Expect = 0.0, Identities = 1059/1343 (78%)

30

>gb|AY206718.1| Homo sapiens peroxisome proliferative activated receptor, alpha (PPARalpha) gene, complete cds

Length = 86215, Score = 912 bits (474), Expect = 0.0, Identities = 481/482 (99%), Gaps = 1/482 (0%)

35

>emb|AL078611.1|HSB5E3 Human DNA sequence from clone SC22CB-5E3 on chromosome 22, complete sequence

Length = 40981, Score = 912 bits (474), Expect = 0.0, Identities = 481/482 (99%), Gaps = 1/482 (0%)

5

>emb|Y16186.1|HSY16186 Homo sapiens mRNA for peroxisome proliferator-activated receptoralpha, partial

Length = 475, Score = 908 bits (472), Expect = 0.0, Identities = 474/475 (99%)

10 >emb|AJ011515.1|CNI011515 Crocodylus niloticus mRNA for peroxisome proliferator activatedreceptor alpha, partial

Length = 984, Score = 810 bits (421), Expect = 0.0, Identities = 789/973 (81%)

15 >gb|M84161.1|XELPPARA Xenopus laevis peroxisome proliferator activated receptor alpha(PPARa) mRNA, complete cds

Length = 3250, Score = 742 bits (386), Expect = 0.0, Identities = 857/1090 (78%), Gaps = 3/1090 (0%)

20 >gb|BC060004.1| Xenopus laevis peroxisome proliferator activated receptor alpha, mRNA(cDNA clone MGC:68537 IMAGE:4031120), complete cds

Length = 3355, Score = 737 bits (383), Expect = 0.0, Identities = 856/1090 (78%), Gaps = 3/1090 (0%)

25 >gb|AF013264.1|AF013264 Oryctolagus cuniculus peroxisome proliferator-activatedreceptor-alpha (PPAR-alpha) mRNA, partial cds

Length = 524, Score = 725 bits (377), Expect = 0.0, Identities = 475/524 (90%)

>emb|AJ007664.1|GGA7664 Gallus gallus mRNA for peroxisome proliferator activated receptoralpha

30 Length = 593, Score = 575 bits (299), Expect = e-161, Identities = 495/593 (83%)

>emb|X75293.1|MMPARA7 Mus musculus PPAR alpha gene, exon 7

Length = 468, Score = 510 bits (265), Expect = e-141, Identities = 387/448 (86%)

>gb|AF175309.1|AF175309 Sus scrofa peroxisome proliferator activated receptor alpha mRNA, partial cds

Length = 515, Score = 494 bits (257), Expect = e-136, Identities = 429/515 (83%)

5 >emb|AL049856.1|HS695O20A Human DNA sequence from clone RP4-695O20 on chromosome 22q13.1-13.33 Contains part of the PPARA gene for Peroxisome Proliferative Activated Receptor Alpha, ESTs, an STS, a GSS and a putative CpG island, complete sequence  
Length = 43497, Score = 464 bits (241), Expect = e-127, Identities = 248/249 (99%), Gaps = 1/249 (0%)

10 >emb|Y16420.1|OCU16420 Oryctolagus cuniculus mRNA for peroxisome proliferator-activated receptor alpha, partial  
Length = 328, Score = 312 bits (162), Expect = 2e-81, Identities = 222/252 (88%)

15 >gb|AY198322.1| Carassius auratus peroxisome proliferator activated receptor alphanRNA, partial cds  
Length = 1049, Score = 269 bits (140), Expect = 8e-69, Identities = 437/583 (74%), Gaps = 3/583 (0%)

20 >emb|X75292.1|MMPARA6 Mus musculus PPAR alpha gene, exon 6  
Length = 223, Score = 269 bits (140), Expect = 8e-69, Identities = 178/197 (90%)

>emb|X75294.1|MMPARA8 Mus musculus PPAR alpha gene, exon 8  
Length = 895, Score = 267 bits (139), Expect = 3e-68, Identities = 213/250 (85%)

25 >gb|AF342937.1|AF342937 Danio rerio peroxisome proliferator-activated receptor beta 1 mRNA,partial cds  
Length = 990, Score = 235 bits (122), Expect = 2e-58, Identities = 432/587 (73%)

30 >emb|X75289.1|MMPARA3 Mus musculus PPAR alpha gene, exon 3  
Length = 267, Score = 206 bits (107), Expect = 1e-49, Identities = 173/206 (83%)

>emb|X89577.1|MMPPARGEN M.musculus mRNA for PPAR alpha protein  
Length = 490, Score = 200 bits (104), Expect = 6e-48, Identities = 150/173 (86%)

35 >emb|AJ416953.1|SSA416953 Salmo salar mRNA for peroxisomal proliferator-activated receptorbeta1 (ppar beta1 gene)  
Length = 1462, Score = 194 bits (101), Expect = 3e-46, Identities = 271/356 (76%)

>gb|AF342945.1|AF342945 Salmo salar peroxisome proliferator-activated receptor beta mRNA,partial cds

Length = 1012, Score = 187 bits (97), Expect = 6e-44, Identities = 261/343 (76%)

5

>emb|AJ243131.1|CNI243131 Crocodylus niloticus partial mRNA for peroxisome proliferatoractivated receptor beta (ppar gene)

Length = 912, Score = 185 bits (96), Expect = 2e-43, Identities = 399/548 (72%), Gaps = 3/548 (0%)

10

>dbj|AK089913.1| Mus musculus melanocyte cDNA, RIKEN full-length enriched library, clone:G270140L11 product:peroxisome proliferator activator receptor delta, full insert sequence

Length = 1740, Score = 183 bits (95), Expect = 9e-43, Identities = 189/236 (80%)

15

>gb|L28116.1|MUSPPARA Mus musculus peroxisome proliferator-activated receptor mRNA, complete cds

Length = 1542, Score = 183 bits (95), Expect = 9e-43, Identities = 189/236 (80%)

>gb|U10375.1|MMU10375 Mus musculus peroxisome proliferator-activated receptor delta mRNA,

20

complete cds

Length = 1323, Score = 183 bits (95), Expect = 9e-43, Identities = 189/236 (80%).

### Example 2: Expression profiling

Total cellular RNA was isolated from cells by one of two standard methods: 1) guanidine 25 isothiocyanate/Cesium chloride density gradient centrifugation [Kellogg, (1990)]; or with the Tri-Reagent protocol according to the manufacturer's specifications (Molecular Research Center, Inc., Cincinnati, Ohio). Total RNA prepared by the Tri-reagent protocol was treated with DNase I to remove genomic DNA contamination.

For relative quantitation of the mRNA distribution of PPARA, total RNA from each cell or tissue 30 source was first reverse transcribed. 85 µg of total RNA was reverse transcribed using 1 µmole random hexamer primers, 0.5 mM each of dATP, dCTP, dGTP and dTTP (Qiagen, Hilden, Germany), 3000 U RnaseQut (Invitrogen, Groningen, Netherlands) in a final volume of 680 µl. The first strand synthesis buffer and Omniscript reverse transcriptase (2 u/µl) were from (Qiagen, Hilden, Germany). The reaction was incubated at 37°C for 90 minutes and cooled on ice. The

volume was adjusted to 6800  $\mu$ l with water, yielding a final concentration of 12.5 ng/ $\mu$ l of starting RNA.

For relative quantitation of the distribution of PPARA mRNA in cells and tissues the Applied Biosystems 7900 HT Sequence Detection system or Biorad iCycler was used according to the manufacturer's specifications and protocols. PCR reactions were set up to quantitate PPARA and the housekeeping genes HPRT (hypoxanthine phosphoribosyltransferase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase),  $\beta$ -actin, and others. Forward and reverse primers and probes for PPARA were designed using the Perkin Elmer ABI Primer Express<sup>TM</sup> software and were synthesized by TibMolBiol (Berlin, Germany). The PPARA forward primer sequence was: 5 Primer1 (SEQ ID NO: 3). The PPARA reverse primer sequence was Primer2 (SEQ ID NO: 4). Probe1 (SEQ ID NO: 5), labelled with FAM (carboxyfluorescein succinimidyl ester) as the reporter dye and TAMRA (carboxytetramethylrhodamine) as the quencher, is used as a probe for PPARA. The following reagents were prepared in a total of 25  $\mu$ l : 1x TaqMan buffer A, 5.5 mM MgCl<sub>2</sub>, 200 nM of dATP, dCTP, dGTP, and dUTP, 0.025 U/ $\mu$ l AmpliTaq Gold<sup>TM</sup>, 0.01 U/ $\mu$ l 10 AmpErase and Probe1 (SEQ ID NO: 5), PPARA forward and reverse primers each at 200 nM, 15 200 nM PPARA FAM/TAMRA-labelled probe, and 5  $\mu$ l of template cDNA. Thermal cycling parameters were 2 min at 50°C, followed by 10 min at 95°C, followed by 40 cycles of melting at 95°C for 15 sec and annealing/extending at 60°C for 1 min.

*Calculation of corrected CT values*

20 The CT (threshold cycle) value is calculated as described in the "Quantitative determination of nucleic acids" section. The CF-value (factor for threshold cycle correction) is calculated as follows :

1. PCR reactions were set up to quantitate the housekeeping genes (HKG) for each cDNA sample.
- 25 2. CT<sub>HKG</sub>-values (threshold cycle for housekeeping gene) were calculated as described in the "Quantitative determination of nucleic acids" section.
3. CT<sub>HKG</sub>-mean values (CT mean value of all HKG tested on one cDNAs) of all HKG for each cDNA are calculated (n = number of HKG):

$$\text{CT}_{\text{HKG-}n}\text{-mean value} = (\text{CT}_{\text{HKG1}}\text{-value} + \text{CT}_{\text{HKG2}}\text{-value} + \dots + \text{CT}_{\text{HKG-}n}\text{-value}) / n$$

4.  $CT_{\text{panel}} \text{ mean value (CT mean value of all HKG in all tested cDNAs)} =$   
 $(CT_{\text{HKG1}}\text{-mean value} + CT_{\text{HKG2}}\text{-mean value} + \dots + CT_{\text{HKG-y}}\text{-mean value}) / y$   
 $(y = \text{number of cDNAs})$

5.  $CF_{\text{cDNA-n}} \text{ (correction factor for cDNA n)} = CT_{\text{panel}}\text{-mean value} - CT_{\text{HKG-n}}\text{-mean value}$

5 6.  $CT_{\text{cDNA-n}} \text{ (CT value of the tested gene for the cDNA n)} + CF_{\text{cDNA-n}} \text{ (correction factor for cDNA n)} = CT_{\text{cor-cDNA-n}} \text{ (corrected CT value for a gene on cDNA n)}$

*Calculation of relative expression*

Definition : highest  $CT_{\text{cor-cDNA-n}} \neq 40$  is defined as  $CT_{\text{cor-cDNA}}$  [high]

Relative Expression =  $2^{(CT_{\text{cor-cDNA}}[\text{high}] - CT_{\text{cor-cDNA-n}})}$

10 *Tissues*

The expression of PPARA was investigated in the tissues listed in table 1.

*Expression profile*

The results of the the mRNA-quantification (expression profiling) is shown in Table 1.

*Table 1: Relative expression of PPARA in various human tissues*

fetal heart	115
heart	163
pericardium	104
heart atrium (right)	452
heart atrium (left)	249
heart ventricle (left)	50
heart ventricle (right)	12
heart apex	449
Purkinje fibers	622
interventricular septum	434
fetal aorta	1
aorta	5
artery	1
coronary artery	6
pulmonary artery	5
carotid artery	5

mesenteric artery	14
vein	2
pulmonic valve	205
coronary artery smooth muscle primary cells	10
HUVEC cells	8
skin	106
adrenal gland	76
thyroid	75
thyroid tumor	34
pancreas	49
pancreas liver cirrhosis	43
esophagus	13
esophagus tumor	79
stomach	49
stomach tumor	117
colon	60
colon tumor	21
small intestine	123
ileum	249
ileum tumor	17
ileum chronic inflammation	37
rectum	123
salivary gland	30
fetal liver	159
liver	399
liver liver cirrhosis	70
liver tumor	391
HEP G2 cells	229
leukocytes (peripheral blood)	23
Jurkat (T-cells)	19
bone marrow	11
erythrocytes	8

lymphnode	9
thymus	42
thrombocytes	4
bone marrow stromal cells	99
bone marrow CD71+ cells	12
bone marrow CD33+ cells	61
bone marrow CD34+ cells	24
bone marrow CD15+ cells	14
cord blood CD71+ cells	10
cord blood CD34+ cells	138
neutrophils cord blood	115
neutrophils peripheral blood	263
spleen	34
spleen liver cirrhosis	24
skeletal muscle	184
adipose	52
fetal brain	29
brain	33
Alzheimer brain	90
cerebellum	3
cerebellum (right)	115
cerebellum (left)	103
cerebral cortex	132
Alzheimer cerebral cortex	73
frontal lobe	120
Alzheimer brain frontal lobe	131
occipital lobe	98
parietal lobe	77
temporal lobe	100
precentral gyrus	74
postcentral gyrus	2
tonsilla cerebelli	50
vermis cerebelli	48
pons	140

substantia nigra	194
cerebral meninges	25
cerebral peduncles	36
corpus callosum	182
hippocampus	124
thalamus	117
dorsal root ganglia	9
spinal cord	92
neuroblastoma SK-N-MC cells	18
neuroblastoma SH-SY5Y cells	108
neuroblastoma IMR32 cells	33
glial tumor H4 cells	38
glial tumor H4 cells + APP	128
HEK CNS	345
HEK CNS + APP	704
retina	123
 fetal lung	124
fetal lung fibroblast IMR-90 cells	63
fetal lung fibroblast MRC-5 cells	81
lung	28
lung right upper lobe	47
lung right mid lobe	25
lung right lower lobe	38
lung lupus disease	98
lung tumor	11
lung COPD	5
trachea	32
 cervix	12
testis	13
HeLa cells (cervix tumor)	24
placenta	21
uterus	38
uterus tumor	76
ovary	212

ovary tumor	116
breast	130
breast tumor	37
MDA MB 231 cells (breast tumor)	23
mammary gland	44
prostate	41
prostate BPH	7
bladder	58
ureter	132
penis	26
corpus cavernosum	13
fetal kidney	172
kidney	372
kidney tumor	92
HEK 293 cells	26

### **Example 3: Antisense Analysis**

Knowledge of the correct, complete cDNA sequence coding for PPARA enables its use as a tool for antisense technology in the investigation of gene function. Oligonucleotides, cDNA or 5 genomic fragments comprising the antisense strand of a polynucleotide coding for PPARA are used either in vitro or in vivo to inhibit translation of the mRNA. Such technology is now well known in the art, and antisense molecules can be designed at various locations along the nucleotide sequences. By treatment of cells or whole test animals with such antisense sequences, the gene of interest is effectively turned off. Frequently, the function of the gene is ascertained by observing 10 behavior at the intracellular, cellular, tissue or organismal level (e.g., lethality, loss of differentiated function, changes in morphology, etc.).

In addition to using sequences constructed to interrupt transcription of a particular open reading frame, modifications of gene expression is obtained by designing antisense sequences to intron regions, promoter/enhancer elements, or even to trans-acting regulatory genes.

### **15 Example 4: Expression of PPARA**

Expression of PPARA is accomplished by subcloning the cDNAs into appropriate expression vectors and transfecting the vectors into expression hosts such as, e.g., *E. coli*. In a particular case,

the vector is engineered such that it contains a promoter for  $\beta$ -galactosidase, upstream of the cloning site, followed by sequence containing the amino-terminal Methionine and the subsequent seven residues of  $\beta$ -galactosidase. Immediately following these eight residues is an engineered bacteriophage promoter useful for artificial priming and transcription and for providing a number 5 of unique endonuclease restriction sites for cloning.

Induction of the isolated, transfected bacterial strain with Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) using standard methods produces a fusion protein corresponding to the first seven residues of  $\beta$ -galactosidase, about 15 residues of "linker", and the peptide encoded within the cDNA. Since 10 cDNA clone inserts are generated by an essentially random process, there is probability of 33% that the included cDNA will lie in the correct reading frame for proper translation. If the cDNA is not in the proper reading frame, it is obtained by deletion or insertion of the appropriate number of bases using well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or the inclusion of an oligonucleotide linker of appropriate length.

The PPARA cDNA is shuttled into other vectors known to be useful for expression of proteins in 15 specific hosts. Oligonucleotide primers containing cloning sites as well as a segment of DNA (about 25 bases) sufficient to hybridize to stretches at both ends of the target cDNA is synthesized chemically by standard methods. These primers are then used to amplify the desired gene segment 20 by PCR. The resulting gene segment is digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternately, similar gene segments are produced by digestion of the cDNA with appropriate restriction enzymes. Using appropriate primers, segments of coding sequence from more than one gene are ligated together and cloned in appropriate vectors. It is possible to optimize expression by construction of such chimeric 25 sequences.

Suitable expression hosts for such chimeric molecules include, but are not limited to, mammalian 25 cells such as Chinese Hamster Ovary (CHO) and human 293 cells., insect cells such as Sf9 cells, yeast cells such as *Saccharomyces cerevisiae* and bacterial cells such as *E. coli*. For each of these cell systems, a useful expression vector also includes an origin of replication to allow propagation in bacteria, and a selectable marker such as the  $\beta$ -lactamase antibiotic resistance gene to allow 30 plasmid selection in bacteria. In addition, the vector may include a second selectable marker such as the neomycin phosphotransferase gene to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts require RNA processing elements such as 3' polyadenylation sequences if such are not part of the cDNA of interest.

Additionally, the vector contains promoters or enhancers which increase gene expression. Such promoters are host specific and include MMTV, SV40, and metallothionein promoters for CHO

cells; trp, lac, tac and T7 promoters for bacterial hosts; and alpha factor, alcohol oxidase and PGH promoters for yeast. Transcription enhancers, such as the rous sarcoma virus enhancer, are used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced PPARA are recovered from the conditioned medium and analyzed using chromatographic methods known in the art. For example, PPARA can be cloned into the expression vector pcDNA3, as exemplified herein. This product can be used to transform, for example, HEK293 or COS by methodology standard in the art. Specifically, for example, using Lipofectamine (Gibco BRL catalog no. 18324-020) mediated gene transfer.

10 **Example 5: Isolation of Recombinant PPARA**

PPARA is expressed as a chimeric protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals [Appa Rao, 1997] and the domain utilized in the FLAGS extension/affinity 15 purification system (Immunex Corp., Seattle, Washington). The inclusion of a cleavable linker sequence such as Factor Xa or enterokinase (Invitrogen, Groningen, The Netherlands) between the purification domain and the PPARA sequence is useful to facilitate expression of PPARA.

**Example 6: Production of PPARA Specific Antibodies**

Two approaches are utilized to raise antibodies to PPARA, and each approach is useful for 20 generating either polyclonal or monoclonal antibodies. In one approach, denatured protein from reverse phase HPLC separation is obtained in quantities up to 75 mg. This denatured protein is used to immunize mice or rabbits using standard protocols; about 100 µg are adequate for immunization of a mouse, while up to 1 mg might be used to immunize a rabbit. For identifying mouse hybridomas, the denatured protein is radioiodinated and used to screen potential murine B-cell hybridomas for those which produce antibody. This procedure requires only small quantities 25 of protein, such that 20 mg is sufficient for labeling and screening of several thousand clones.

In the second approach, the amino acid sequence of an appropriate PPARA domain, as deduced 30 from translation of the cDNA, is analyzed to determine regions of high antigenicity. Oligopeptides comprising appropriate hydrophilic regions are synthesized and used in suitable immunization protocols to raise antibodies. The optimal amino acid sequences for immunization are usually at the C-terminus, the N-terminus and those intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the protein is in its natural conformation.

Typically, selected peptides, about 15 residues in length, are synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH; Sigma, St. Louis, MO) by reaction with M-maleimidobenzoyl-N-hydroxy-succinimide ester, MBS. If necessary, a cysteine is introduced at the N-terminus of the peptide to 5 permit coupling to KLH. Rabbits are immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity by binding the peptide to plastic, blocking with 1% bovine serum albumin, reacting with antisera, washing and reacting with labeled (radioactive or fluorescent), affinity purified, specific goat anti-rabbit IgG.

Hybridomas are prepared and screened using standard techniques. Hybridomas of interest are 10 detected by screening with labeled PPARA to identify those fusions producing the monoclonal antibody with the desired specificity. In a typical protocol, wells of plates (FAST; Becton-Dickinson, Palo Alto, CA) are coated during incubation with affinity purified, specific rabbit anti-mouse (or suitable antispecies 1 g) antibodies at 10 mg/ml. The coated wells are blocked with 1% bovine serum albumin, (BSA), washed and incubated with supernatants from hybridomas. After 15 washing the wells are incubated with labeled PPARA at 1 mg/ml. Supernatants with specific antibodies bind more labeled PPARA than is detectable in the background. Then clones producing specific antibodies are expanded and subjected to two cycles of cloning at limiting dilution. Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody is purified from mouse ascitic fluid by affinity chromatography on Protein A. 20 Monoclonal antibodies with affinities of at least

$10^8 \text{ M}^{-1}$ , preferably  $10^9$  to  $10^{10} \text{ M}^{-1}$  or stronger, are typically made by standard procedures.

#### **Example 7: Diagnostic Test Using PPARA Specific Antibodies**

Particular PPARA antibodies are useful for investigating signal transduction and the diagnosis of infectious or hereditary conditions which are characterized by differences in the amount or 25 distribution of PPARA or downstream products of an active signaling cascade.

Diagnostic tests for PPARA include methods utilizing antibody and a label to detect PPARA in human body fluids, membranes, cells, tissues or extracts of such. The polypeptides and antibodies of the present invention are used with or without modification. Frequently, the polypeptides and antibodies are labeled by joining them, either covalently or noncovalently, with a substance which 30 provides for a detectable signal. A wide variety of labels and conjugation techniques are known and have been reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, chromogenic agents, magnetic particles and the like.

A variety of protocols for measuring soluble or membrane-bound PPAR $\alpha$ , using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on PPAR $\alpha$  is preferred, but a competitive binding assay 5 may be employed.

**Example 8: Purification of Native PPAR $\alpha$  Using Specific Antibodies**

Native or recombinant PPAR $\alpha$  is purified by immunoaffinity chromatography using antibodies 10 specific for PPAR $\alpha$ . In general, an immunoaffinity column is constructed by covalently coupling the anti-TRH antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared from immune sera either by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia LKB Biotechnology, Piscataway N.J.). Likewise, monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified 15 immunoglobulin is covalently attached to a chromatographic resin such as CnBr-activated Sepharose (Pharmacia LKB Biotechnology). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such immunoaffinity columns are utilized in the purification of PPAR $\alpha$  by preparing a fraction 20 from cells containing PPAR $\alpha$  in a soluble form. This preparation is derived by solubilization of whole cells or of a subcellular fraction obtained via differential centrifugation (with or without addition of detergent) or by other methods well known in the art. Alternatively, soluble PPAR $\alpha$  containing a signal sequence is secreted in useful quantity into the medium in which the cells are grown.

A soluble PPAR $\alpha$ -containing preparation is passed over the immunoaffinity column, and the 25 column is washed under conditions that allow the preferential absorbance of PPAR $\alpha$  (e.g., high ionic strength buffers in the presence of detergent). Then, the column is eluted under conditions that disrupt antibody/protein binding (e.g., a buffer of pH 2-3 or a high concentration of a chaotrope such as urea or thiocyanate ion), and PPAR $\alpha$  is collected.

**Example 9: Drug Screening**

30 This invention is particularly useful for screening therapeutic compounds by using PPAR $\alpha$  or binding fragments thereof in any of a variety of drug screening techniques. As PPAR $\alpha$  is a Nuclear Receptor any of the methods commonly used in the art may potentially be used to identify

PPARA ligands. For example, the activity of a Nuclear Receptor such as PPARA can be measured using any of a variety of appropriate functional assays in which activation of the receptor results in an observable change in the level of a reportergene which is transcriptionally activated by the Nuclear Receptor. Alternatively, the polypeptide or fragment employed in such a test is either free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the polypeptide or fragment. Drugs are screened against such transformed cells in competitive binding assays. Such cells, either in viable or fixed form, are used for standard binding assays.

10 Measured, for example, is the formation of complexes between PPARA and the agent being tested. Alternatively, one examines the diminution in complex formation between PPARA and a ligand caused by the agent being tested.

Thus, the present invention provides methods of screening for drug candidates, drugs, or any other agents which affect signal transduction. These methods, well known in the art, comprise 15 contacting such an agent with PPARA polypeptide or a fragment thereof and assaying (i) for the presence of a complex between the agent and PPARA polypeptide or fragment, or (ii) for the presence of a complex between PPARA polypeptide or fragment and the cell. In such competitive binding assays, the PPARA polypeptide or fragment is typically labeled. After suitable incubation, free PPARA polypeptide or fragment is separated from that present in bound form, and the amount 20 of free or uncomplexed label is a measure of the ability of the particular agent to bind to PPARA or to interfere with the PPARA-agent complex.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to PPARA polypeptides. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other 25 surface. The peptide test compounds are reacted with PPARA polypeptide and washed. Bound PPARA polypeptide is then detected by methods well known in the art. Purified PPARA are also coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies are used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which 30 neutralizing antibodies capable of binding PPARA specifically compete with a test compound for binding to PPARA polypeptides or fragments thereof. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic determinants with PPARA.

**Example 10: Rational Drug Design**

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact, agonists, antagonists, or inhibitors. Any of these examples are used to fashion drugs which are more active or stable forms of the

5 polypeptide or which enhance or interfere with the function of a polypeptide in vivo.

In one approach, the three-dimensional structure of a protein of interest, or of a protein-inhibitor complex, is determined by x-ray crystallography, by computer modeling or, most typically, by a combination of the two approaches. Both the shape and charges of the polypeptide must be ascertained to elucidate the structure and to determine active site(s) of the molecule. Less often,

10 useful information regarding the structure of a polypeptide is gained by modeling based on the structure of homologous proteins. In both cases, relevant structural information is used to design efficient inhibitors. Useful examples of rational drug design include molecules which have improved activity or stability or which act as inhibitors, agonists, or antagonists of native peptides.

It is also possible to isolate a target-specific antibody, selected by functional assay, as described

15 above, and then to solve its crystal structure. This approach, in principle, yields a pharmacore upon which subsequent drug design is based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids is expected to be an analog of the original receptor. The anti-id is then used to identify and isolate peptides from  
20 banks of chemically or biologically produced peptides. The isolated peptides then act as the pharmacore.

By virtue of the present invention, sufficient amount of polypeptide are made available to perform such analytical studies as X-ray crystallography. In addition, knowledge of the PPARA amino acid sequence provided herein provides guidance to those employing computer modeling

25 techniques in place of or in addition to x-ray crystallography.

**Example 11: Identification of Other Members of the Signal Transduction Complex**

The inventive purified PPARA is a research tool for identification, characterization and purification of other signal transduction pathway proteins. Radioactive labels are incorporated into a selected PPARA domain by various methods known in the art and used in vitro to capture

30 interacting molecules. A preferred method involves labeling the primary amino groups in PPARA with <sup>125</sup>I Bolton-Hunter reagent. This reagent has been used to label various molecules without concomitant loss of biological activity.

Labeled PPARA is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, membrane-bound PPARA is covalently coupled to a chromatography column. Cell-free extract derived from synovial cells or putative target cells is passed over the column, and molecules with appropriate affinity bind to PPARA. PPARA-  
5 complex is recovered from the column, and the PPARA-binding ligand disassociated and subjected to N-terminal protein sequencing. The amino acid sequence information is then used to identify the captured molecule or to design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

In an alternate method, antibodies are raised against PPARA, specifically monoclonal antibodies.  
10 The monoclonal antibodies are screened to identify those which inhibit the binding of labeled PPARA. These monoclonal antibodies are then used therapeutically.

**Example 12: Use and Administration of Antibodies, Inhibitors, or Antagonists**

Antibodies, inhibitors, or antagonists of PPARA or other treatments and compounds that are limiters of signal transduction (LSTs), provide different effects when administered therapeutically.  
15 LSTs are formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although pH may vary according to the characteristics of the antibody, inhibitor, or antagonist being formulated and the condition to be treated. Characteristics of LSTs include solubility of the molecule, its half-life and antigenicity/immunogenicity. These and other characteristics aid in defining an effective carrier.  
20 Native human proteins are preferred as LSTs, but organic or synthetic molecules resulting from drug screens are equally effective in particular situations.

LSTs are delivered by known routes of administration including but not limited to topical creams and gels; transmucosal spray and aerosol; transdermal patch and bandage; injectable, intravenous and lavage formulations; and orally administered liquids and pills particularly formulated to resist stomach acid and enzymes. The particular formulation, exact dosage, and route of administration  
25 is determined by the attending physician and varies according to each specific situation.

Such determinations are made by considering multiple variables such as the condition to be treated, the LST to be administered, and the pharmacokinetic profile of a particular LST. Additional factors which are taken into account include severity of the disease state, patient's age,  
30 weight, gender and diet, time and frequency of LST administration, possible combination with other drugs, reaction sensitivities, and tolerance/response to therapy. Long acting LST formulations might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular LST.

Normal dosage amounts vary from 0.1 to  $10^5$   $\mu$ g, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212. Those skilled in the art employ different formulations for different LSTs. Administration to cells such as nerve cells 5 necessitates delivery in a manner different from that to other cells such as vascular endothelial cells.

It is contemplated that abnormal signal transduction, trauma, or diseases which trigger PPARA activity are treatable with LSTs. These conditions or diseases are specifically diagnosed by the tests discussed above, and such testing should be performed in suspected cases of viral, bacterial or 10 fungal infections, allergic responses, mechanical injury associated with trauma, hereditary diseases, lymphoma or carcinoma, or other conditions which activate the genes of lymphoid or neuronal tissues.

#### **Example 13: Production of Non-human Transgenic Animals**

Animal model systems which elucidate the physiological and behavioral roles of the PPARA are 15 produced by creating nonhuman transgenic animals in which the activity of the PPARA is either increased or decreased, or the amino acid sequence of the expressed PPARA is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a PPARA, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriately 20 fertilized embryos in order to produce a transgenic animal or 2) homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these PPARA sequences. The technique of homologous recombination is well known in the art. It replaces the native gene 25 with the inserted gene and hence is useful for producing an animal that cannot express native PPARAs but does express, for example, an inserted mutant PPARA, which has replaced the native PPARA in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and the technique is useful for producing an animal which expresses its own and added PPARA, resulting in overexpression of the PPARA.

30 One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as cesiumchloride M2 medium. DNA or cDNA encoding PPARA is purified from a vector by methods well known to the one skilled in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental

means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the transgene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a piper puller) and the egg to be injected is put in 5 a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse which is a mouse stimulated by the appropriate hormones in order to maintain false pregnancy, where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg but is used here only for exemplary purposes.

10 **References**

U.S. 4,522,811  
U.S. 5,283,317  
U.S. 5,565,332  
WO 93/03151  
15 WO 94/13804  
WO 01/20037  
U.S. 6,544,743  
U.S. 6,689,574  
Altschul SF et al.,; Nucleic Acids Res 1997 Sep 1; 25(17): 3389-402  
20 Appa Rao et al., 1997, Protein Expr Purif Nov, 11(2): 201-8  
Barnes, 2000, Chest, 117:10S14S  
Berger et al., 1992, J Steroid Biochem Mol Biol. 41 (3-8): 733-738  
Botstein et al., 1980 , Am J Hum Genet. 32: 314-31  
Bourguet et al., 2000, Trends Pharmacol. Sci. 21: 381-388  
25 Charles et al., 2000, J Appl Toxicol. 20, 6: 449-454  
Colbere-Garapin et al., 1981, *J. Mol. Biol.* 150, 1-14  
Engelhard et al., 1994, *Proc. Nat. Acad. Sci.* 91, 3224-3227  
Gergen and Weiss , 1992, Am Rev Respir Dis 146:823-824  
Gibson et al., 1996, Genome Research 6: 995-1001  
30 Gutendorf, B. and Westendorf, J.(2001) Toxicology 166: 79-89  
Haseloff et al., 1988 , *Nature* 334, 585-591  
Heid et al., 1996, Genome Research 6: 986-994  
Holland et al., 1991, PNAS 88: 7276-7280  
Iwabuchi et al., 1993, *Oncogene* 8, 1693-1696  
35 Jeffreys et al., 1985, Nature 316: 76-9

Johnson *et al.*, 1989, *Endoc. Rev.* 10, 317-331

Kellogg *et al.*, 1990, *Anal. Biochem.* 189:202-208

Kersten *et al.* (2000), *Nature* 405: 421-424

Lam , 1997, *Anticancer Drug Res.* 12(3):145-67

5 Lee *et al.*, 1998, *Mol Endocrinol.* 12(8): 1184-1192

Livak *et al.*, 1995 , *PCR Methods and Applications* 357-362

Logan, Shenk, 1984, *Proc. Natl. Acad. Sci.* 81, 3655-3659

Lowy *et al.*, 1980, *Cell* 22, 817-23

Maddox *et al.*, 1983, *J. Exp. Med.* 158, 1211-1216

10 McConnell *et al.* , 1992 , *Science* 257, 1906-1912

Nicholls *et al.*, 1993, *J. Immunol. Meth.* 165, 81-91

Nichols *et al.*, 1998, *Anal Biochem.* 257: 112-119

Olefsky & Jerrold M., (2001) *J Biol Chem*, Vol. 276, No.40 Issue 5: 36863-36864

Piatak *et al.*, 1993, *BioTechniques* 14:70-81

15 Piatak *et al.*, 1993, *Science* 259:1749-1754

Porath *et al.*, 1992, *Prot. Exp. Purif.* 3, 263-281

Roberge *et al.*, 1995, *Science* 269, 202-204

Robinson-Rechavi *et al.*, (2003) *J Struct Funct Genomics.* 3(1-4):177-84

Rosen J. *et al.*, 2003, *Drug Discovery & Developement* Vol 6 No 2: 224-230

20 Sher *et al.* (1993), *Biochemistry* 32: 5598-5604

Sjolander, Urbaniczky, 1991, *Anal. Chem.* 63, 2338-2345

Szabo *et al.*, 1995, *Curr. Opin. Struct. Biol.* 5, 699-705

Thomas, 1980, *Proc. Nat. Acad. Sci.*, 77:5201-5205

Uhlmann *et al.*, 1987, *Tetrahedron. Lett.* 215, 3539-3542

25 Weber *et al.*, 1990, *Genomics* 7: 524-30

Wigler *et al.*, 1977, *Cell* 11, 223-32

Wigler *et al.*, 1980, *Proc. Natl. Acad. Sci.* 77, 3567-70